SCREENING DISINFECTION BYPRODUCTS AND PHENOLIC COMPOUNDS FOR ESTROGENIC ACTIVITY

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ABSTRACT

PAUL EBOHON: Screening Disinfection Byproducts and Phenolic Compounds for Estrogenic Activity
(Under the direction of Howard S. Weinberg and Rebecca Fry)

Very few of the more than 600 individual disinfection byproducts (DBPs) identified in drinking water are currently regulated based on their carcinogenic potential. Endocrine disruption is another potential mechanism through which DBPs could affect human health but limited research has evaluated this threat.

This thesis evaluates the potential endocrine activity of several DBPs and phenolic compounds using an *in vitro* yeast estrogen screen. The rank order of tested compounds demonstrates that while estrogenic activity is observed only at concentrations that are two to three orders of magnitude higher than typical environmental concentrations, the unregulated DBPs such as mucochloric acid and dibromoacetonitrile show higher potency than the regulated dibromo- and trichloroacetic acids. It is also clear that the presence of a halogen in the DBP is responsible for the observed estrogenic activity since the non halogenated structures (acetic acid and acetonitrile) showed no activity in the same concentration range.

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2,4-DCP	2,4-dichlorophenol
4-NP	4-Nonylphenol
8-OH-dG	8-hydroxydeoxyguanosine
ACF	Aberrant Crypt Foci
ADME	Absorption, Distribution, Metabolism, and Excretion
ALP	Alkaline Phosphatase
AWWARF	American Water Works Association Research Foundation
BPA	Bisphenol A
$CaCO_3$	Calcium Carbonate
CCL	Contaminant Candidate List
CCL3	Contaminant Candidate List 3
CDC	Centers for Disease Control and Prevention
CHO	Chinese Hamster Ovary
Conc.	Concentration
DBPs	Disinfection By-Products
DDT	Dichlorodiphenyltrichloroethane
DEPA	Danish Environmental Protection Agency
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
DWS	Drinking Water Strategy
DWTPs	Drinking Water Treatment Plants
E1	Estrone

E2	17-β-Estradiol
E3	Estriol
EC_{10}	Effective Concentration$_{10}$
EC_{50}	Effective Concentration$_{50}$
EDCs	Endocrine Disrupting Compounds
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE2	17-α-ethinylestradiol
EEQ	Estradiol Equivalents
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
ER-CALUX	Estrogen Receptor-mediated Chemical-Activated Luciferase gene Expression assay
ERE	Estrogen Response Elements
FQPA	Food Quality Protection Act
HAA5	Sum of 5 Haloacetic acids
HAA9	Sum of 9 Haloacetic acids
hERα	Human Estrogen Receptor alpha
hERβ	Human Estrogen Receptor beta
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography with Ultraviolet detector
ICR	Information Collection Rule
ICRDA	Information Collection Rule Data Analysis
IRIS	Integrated Risk Information System

IUGR	Intrauterine Growth Restriction
LGW	Laboratory Grade Water
LOD	Limit of Detection
Max	Maximum
MCL	Maximum Contaminant Level
MCLGs	Maximum Contaminant Level Goals
MDA	Malondialdehyde
MELN	MCF-7-ERE-βGlob-Luc-Neo
mRNA	Messenger Ribonucleic Acid
MX	3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone
NCI	National Cancer Institute
NCRI	National Cancer Research Institute
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
NDPA	N-nitroso-di-n-propylamine
NIEHS	National Institute of Environmental Health Sciences
NOM	Natural Organic Matter
NOMS	National Organics Monitoring Survey
NOR	National Organics Reconnaissance Survey
NPDWRs	National Primary Drinking Water Regulations
NPYR	N-nitrosopyrrolidine
NSDWRs	National Secondary Drinking Water Regulations
OD	Optical Density

o-NPG	o-Nitrophenyl-β-Galactosidase
OWCs	Organic Wastewater Contaminants
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SAR	Structural Activity Relationship
SDWA	Safe Drinking Water Act
SMCLs	Secondary Maximum Contaminant Levels
STP	Sewage Treatment Plant
THM4	Sum of 4 Trihalomethanes
THMs	Trihalomethanes
TOC	Total Organic Carbon
TOX	Total Organic Halide
TTHMs	Total Trihalomethanes
U.S. EPA	United States Environmental Protection Agency
U.S.	United States
USGS	United States Geological Survey
WHO	World Health Organization
WWTPs	Waste Water Treatment Plants
YES	Yeast Estrogen Screen
βME	2-mercaptoethanol

1.0 INTRODUCTION

The disinfection of drinking water with chlorine in the U.S. has played a major role in the reduction of waterborne diseases that had exacted a heavy toll in illness and deaths prior to its use in 1908. Despite this benefit, the reaction of chlorine with natural organic matter (NOM), bromide, iodide and anthropogenic contaminants in surface and groundwater during treatment generates disinfection by-products (DBPs) (Rook 1974). The presence of these compounds in drinking water has been a health concern over the last 35 years following their discovery and subsequent epidemiological studies associating them with increased risk of bladder cancer, spontaneous abortion, and low birth weight (Villanueva et al., 2007; Waller et al., 1998; Wright et al., 2003). Results from *in vitro* studies using laboratory animals showed that these compounds (administered at elevated concentrations) caused spermatoxicity, developmental abnormalities, fetal death and growth retardation (Linder et al., 1994; Hunter et al., 1996; Smith et al., 1992).

Health concerns associated with DBPs prompted the United States Environmental Protection Agency (U.S. EPA) to regulate 11 of them; although over 600 (with limited studies on their occurrence and health effects) have been identified (Richardson et al., 2007). Utilities having difficulty complying with established regulations normally end up switching to the use of alternate disinfectants such as chloramines, ozone and chlorine dioxide or their

combinations, but this generates a different suite of DBPs, some of which have been shown to be more cytotoxic and genotoxic than their regulated counterparts (Plewa et al., 2007)

Human exposure to complex mixtures of DBPs in drinking water is a public health concern yet very few studies have been conducted to determine if they induce estrogenic activity. Some *in vivo* studies have suggested that chloroform and dibromoacetic acid are suspected endocrine disrupting compounds (EDCs) (Brittebo et al., 1987; Goldman & Murr 2003). Before addressing whole drinking water samples containing complex mixtures of DBPs, it is helpful to generate a comparative database of their estrogenic activity when acting as single compounds. Such information would help determine which DBPs need more study, including occurrence levels and effective treatment technologies to reduce their formation, in order to minimize human health risks associated with their possible estrogenic activity in drinking water.

1.1 Literature Review

1.1.1 Risks of Waterborne Disease

About 1.1 billion people worldwide lack access to safe drinking water, and twice that amount lack access to adequate sanitation (WHO, 2006). As a result of this, the World Health Organization (WHO) estimates that 3.4 million people die every year due to water- borne related diseases and most of these casualties are children (WHO, 2001). Bacteria, viruses and parasitic protozoan are the major categories of pathogens responsible for causing waterborne diseases and are known to contaminate both surface and groundwater (U.S. EPA, 1998a). The waterborne diseases of main concern during the early part of the 20th century in the U.S. were typhoid fever and amebiasis (U.S. EPA, 1993a). In 1900 and 1920, the incidence of typhoid fever in the U.S. was approximately 100 and 34 per 100,000 populations,

respectively. Between 1930 and 1940, the incidence of this waterborne disease decreased by 41 and 60 percent, respectively, and by 1960, approximately 88 percent reduction had been recorded by the Centers for Disease Control and Prevention (CDC, 1999). The introduction of disinfection of public water supplies in the U.S. since 1908 contributed to a significant reduction in all waterborne illnesses in the U.S. including typhoid fever, cholera, and amoebic dysentery (CDC, 1999; Ohanian et al., 1989) to the point where these have essentially been eradicated from public water supplies.

Despite the significant reduction of waterborne diseases and illness resulting from the use of chlorine for disinfection of drinking water, several cases have served as a reminder for the need to continuously provide adequate disinfection and water treatment processes. In Walkerton (Canada), *Escherichia coli* 0157:H7 and *Campylobacter jejuni* bacterial strains found in surface waters were responsible for contaminating drinking water sources with inadequate residual chlorine (Holme 2003). This incidence, that occurred in May, 2000, caused seven deaths and over 2000 illness that could easily have been prevented if the chlorinators in the water sources were adequately maintained and working properly. Another case is that of the Cryptosporidium outbreak in Milwaukee in 1993. The infectious agent that caused this outbreak was *Cryptosporidium parvum* and some of the symptoms associated with cryptosporidiosis include nausea, vomiting, fever and diarrhea (Mackenzie et al., 1994). Infection of humans by *Cryptosporidium parvum* occurs by drinking water contaminated with the oocysts of this organism. Once in the body, it reproduces and can return to the aquatic environment after being excreted from its host. One key feature of the oocyst is its resistance to chlorination; therefore, water systems employing filtration have a better chance of getting rid of them. The outbreak in Milwaukee was due to contamination of the drinking

water source (Lake Michigan) by human sewage and cattle effluent containing the cryptosporidium oocyst. The Milwaukee Water Works failed to properly monitor its water treatment process and this led to more than 400,000 people being infected with cryptosporidiosis (Dillingham et al., 2002).

1.1.2 Drinking Water Treatment and Disinfection

The need for potable drinking water dates back as far as 1500 B.C. During this era, not much was known about the science of how microorganisms in water could cause waterborne diseases and illness. The goal was to have palatable water and processes such as boiling and exposing water to sunlight were used for drinking water treatment (U.S. EPA, 2000). The weakness during this era was that palatable water was assumed to be safe for consumption. In 500 B.C, the first cloth filter bag was invented by Hippocrates, who also believed that palatable water was safe for consumption (Baker & Taras 1981).

Awareness on the need for potable drinking water increased in 1676 when Anthony Von Leeuwenhoek discovered microorganisms in pond water by looking through a microscope (Becker et al., 2009). During this era, awareness of the need for drinking water treatment heightened and individuals employed the use of domestic water filters made from sponges, charcoal, and wool (Baker & Taras 1981). In 1804, the first municipal water treatment plant that supplied water to residents was built in Scotland. This plant and another built in Paris in 1806 mainly employed filtration to treat drinking water (U.S. EPA, 2000; Baker & Taras 1981).

Use of chlorination in the drinking water process did not begin until John Snow linked drinking water to disease in 1855 (U.S. EPA, 2000). While investigating the cases involving cholera outbreaks in municipal areas in England, he discovered that those that had

water supplied from a particular source were more likely impacted. The cholera incidence was linked to sewage contamination of one of the water supplies and Snow stopped the outbreak by persuading the local council to disable the Broad street well pump by removing its handle. Results from this incident as well as discoveries by other scientists that particles in source water could harbor pathogens, prompted use of filtration as well as chlorination in the drinking water treatment process worldwide (U.S. EPA, 2000). In 1908, Jersey City (N.J., U.S.A.) became the first city in the U.S. to apply chlorine as a disinfectant in its water treatment process. Over 1000 U.S. cities were employing chlorination in their drinking water treatment processes by 1918 (U.S. EPA, 2000; Fowle & Kopler 1986). At the present time, the use of chlorine as a means to destroy harmful microorganisms continues to be a major technique in drinking water treatment and is most effective when used following coagulation and sedimentation in combination with filtration (Hitzfeld et al., 2000; Betancourt & Rose 2004).

1.1.3 Disinfection By-Products

Studies on the health effects associated with the use of chlorine during drinking water treatment were not conducted until 50 years after it was initially used as a disinfectant (Fowle & Kopfler 1986). Results from an *in vivo* study conducted in 1968 showed that there were no health effects from the presence of chlorine in drinking water (Drukery 1968), but an article published in 1969 highlighted that chlorine in drinking water could be mutagenic (Lederberg 1969). Apart from inactivating and killing microorganisms that would normally cause waterborne diseases in drinking water, chemical disinfectants containing chlorine are powerful oxidants that react with natural organic matter (NOM), bromide, iodide and other contaminants present in water leading to the formation of disinfection by-products (DBPs).

During the early 1970s, the first class of DBPs known as trihalomethanes (THMs) which includes chloroform was identified (Bellar et al., 1974; Rook 1974).

After the discovery of DBPs in drinking water, subsequent studies were undertaken to determine their possible health effects. The first epidemiological studies conducted in Louisiana showed a correlation between prostrate/breast cancer and chlorinated drinking water received from the Mississippi River (Page et al., 1976). It is important to note that results generated from this study did not address confounding factors such as alcohol and tobacco use by individuals as well as patients with cancer who migrated to the state. A report produced by the National Cancer Research Institute (NCRI) showed that chloroform was a carcinogenic compound that caused kidney and liver tumors in laboratory animals (NCI, 1976) while other studies conducted in the U.S., Israel and Canada using the Salmonella/mammalian microsome assay showed that organic extracts from concentrated chlorinated drinking water were mutagenic (Loper et al., 1978; Nestmann et al., 1979; Glatz et al., 1978). Results from these studies showed that despite the advantages of using chlorine in treating water, its potential harmful health effects could not be ignored.

1.1.4 U.S. Regulation of DBPs

Concerns about the health effects of DBPs in drinking water led the U.S. EPA to establish DBP regulations for water utilities. In 1979, the agency set a maximum contaminant level (MCL) for total trihalomethanes (TTHMs) at 100 μg/L (U.S. EPA, 1979). In 1998, this standard was strengthened by reducing the MCL to 80 μg/L under the U.S. EPA's Stage 1 Disinfectants (D)/DBP Rule. In addition to lowering the MCL for TTHMs, the U.S. EPA's Stage 1 D/DBP Rule also regulated other classes of DBPs each having its own MCL (U.S.

EPA, 1998b). One class included the haloacetic acids, which are generally formed at the next highest concentrations compared to THMs (Singer 2004). The sum of the concentrations of five haloacetic acids (HAA5: bromoacetic, dibromoacetic, chloroacetic, dichloroacetic and trichloroacetic acid) were given a MCL of 60 μg/L. These HAA5 were chosen for regulation based on their available occurrence data. In addition to inadequate occurrence data, the other four chlorine- and bromine-containing haloacetic acids were not included for regulation as adequate methods and standards were unavailable for their measurement at the time of promulgation. Bromate (a DBP formed during ozonation) was regulated at 10 μg/L and chlorite, formed as a result of chlorine dioxide treatment, was regulated at 1000 μg/L. The U.S. EPA Stage 1 DBP Rule required the running annual average of samples collected from a water utility distribution system to be below the MCL for each regulated DBP.

The MCL set for each DBP under the Stage 1 Rule was meant to provide protection against any adverse health effect; however, these limits cannot guarantee protection and risks associated with unidentified DBPs. In order to address this issue, this Rule also stressed the use of enhanced coagulation to improve the removal of NOM in a conventional water treatment plant (U.S. EPA, 1999). This rule applied to all systems using surface and groundwater during conventional drinking water treatment processes involving filtration, coagulation, sedimentation, and softening as well as those using a water source that experience a rapid shift in its water characteristics (such as temperature and turbidity) due to the direct impact of a surface water's conditions. Prior to the first point of disinfection, enhanced coagulation requires removal of a percentage of Total Organic Carbon (TOC) which is used as a surrogate measure of NOM as shown in Table 1.1. The specified percentage of TOC removal is based upon the results of bench, pilot and full scale studies

conducted at a large number of utilities (Chowdhury et al., 1997) and relate to the TOC and alkalinity of the source water. A water source with higher alkalinity requires a lower TOC removal because of the difficulty in using a coagulant to depress the pH of the water into a range between 5.5 and 6.5 (where TOC removal is optimal).

Table 1.1 Required Removal of TOC by Enhanced Coagulation as Specified under Stage 1 Rule

	Source Water Alkalinity (mg/L as $CaCO_3$)		
Source Water TOC (mg/L)	0 to 60	>60 to 120	>120
>2.0 – 4.0	35.0%	25.0%	15.0%
>4.0 - 8.0	45.0%	35.0%	25.0%
>8.0	50.0%	40.0%	30.0%

1.1.5 Evolving DBP Regulation and Implications for Drinking Water Treatment Plants

The Stage 2 D/DBP Rule still requires water utilities to comply with the MCL set by the U.S. EPA for each DBP but involves locations within the utility's distribution system. Under this rule, the running annual average of water samples collected from locations within the distribution system cannot exceed the MCL for each DBP (U.S. EPA, 2006a). This change was made by the U.S. EPA since it was possible that some consumers within a drinking water utility customer base were receiving water with DBPs that exceeded the MCL set forth by the agency, while the utilities were still under compliance based on the Stage 1 Rule. The implementation of the Stage 2 Rule addressed the reduction of peak DBP levels that occurred within distribution systems during high temperatures. Control of these peak levels resulting from regulation ultimately helps to protect the health of water to all consumers located within the distribution systems (Singer 2004).

The changes made by the U.S. EPA based on reduction of the MCL for TTHMs, regulation of other classes of DBPs, and the involvement of locations as specified under the Stage 2 Rule made compliance difficult for water utilities using chlorine for disinfection. In order to address this issue, many utilities switched to using alternate disinfectants such as chloramines, chlorine dioxide and ozone or combinations of them. The use of these alternate disinfectants has been shown to reduce the formation of some of the regulated THMs and HAA5 but they produce other classes of DBPs that might be more carcinogenic and genotoxic than their regulated counterparts (Richardson et al., 2007; Plewa et al., 2007; Plewa et al., 2002). For example, chloramines form reduced levels of haloacetic acids (Singer 2004) but higher levels of N-nitrosodimethylamine (NDMA) which has been shown to be carcinogenic in studies using laboratory animals (U.S. EPA, 1993b). Iodoacetic acid is another DBP of concern that is found in waters treated with chloramines (Weinberg et al., 2011). A study using mammalian cells has shown that this DBP is the most potent in terms of genotoxic activity (Plewa et al., 2004). The use of alternate disinfectants also produces DBPs whose health effects remain unknown. Chloropicrin, which can be produced during ozonation followed by chlorination (Merlet et al., 1985; Hoigne & Bader 1988), is an example of such a DBP with no carcinogenic activity data listed under the U.S. EPA Integrated Risk Information System (IRIS).

1.1.6 Presence of DBPs in Drinking Water Treatment Plants

Various studies have been conducted to determine the occurrence levels of DBPs in drinking water treatment plants (DWTPs). Results from early studies conducted from 1975 to 1977 showed the presence of significant concentrations of THMs in tested water samples (Symons et al., 1975; McGuire & Meadow 1988). An occurrence study involving 727

utilities was conducted by the American Water Works Association Research Foundation (AWWARF) in 1987 and all 4 of the regulated THMs (THM4) were detected in finished water (McGuire & Meadow 1988). Another study conducted from 1988 to 1989 involving 35 utilities showed that the trihalomethanes and haloacetic acids accounted for the largest class of DBPs detected in finished water at treatment plants (Krasner et al., 1989) and this result was complemented by those provided under the Information Collection rule (ICR) (McGuire et al., 2002). The THM4 represents the sum of the concentrations of chloroform, bromoform, bromodichloromethane and chlorodibromomethane and is often referred to in the literature as total trihalomethanes (TTHM). The median TTHM levels from early studies including the ICR are summarized in Table 1.2. An important trend is that TTHM levels significantly decreased over the years due to water utilities complying with DBP regulation.

Previous DBP occurrence studies still did not provide enough information on the identity of DBPs and their levels in consumers' drinking water but an EPA priority study involving 12 drinking water treatment plants that employed different treatment options and disinfectants partially addressed this issue (Weinberg et al., 2002). DBP selection for this study was based on results from an extensive Structural Activity Relationships analysis (SAR) that prioritized these chemicals based on their genotoxic and carcinogenic potential (Woo et al., 2002). Results from the SAR analysis showed that the halomethanes, haloacids, haloacetonitriles, haloacetates, haloketones, haloacetaldehydes, halonitromethanes, haloamides, and halogenated furanones were DBPs that needed prioritizing because of their elevated carcinogenic and genotoxic potential. Iodo-THMs are one of these priority DBP groups that were found in one of the drinking water utilities that used chloramines as terminal disinfectant. The median levels of THM4 and HAA9 from the U.S. EPA priority

DBP study as shown in Table 1.3 varied slightly when compared to those from the ICR, while that for chloral hydrate (one of the haloacetaldehydes) was similar. It is important to note that in order to detect the priority DBPs, the 12 treatment plants were targeted because the water sources had high TOC and bromide content. Occurrence levels for selected DBPs from the 12 treatment plants are shown in Table 1.4.

Table 1.2 Snapshots of TTHM Levels in U.S. Drinking Water

Study	Number of Utilities tested	Median Conc. (μg/L)	Reference
[a]NOR	80	41	Symons et al., 1975
[b]NOMS Phase I	111	45	Brass et al., 1997
[c]AWWARF	727	39	McGuire et al., 1988
U.S. EPA	35	36	Krasner et al., 1989
[d]U.S. EPA ICR	500	39	McGuire et al., 2002

[a]NOR: National Organics Reconnaissance Survey; [b]NOMS: National Organics Monitoring Survey [c]AWWARF: American Water Works Association Research Foundation; [d]U.S. EPA ICR: U.S. EPA Information Collection Rule.

Table 1.3 Median Precursors and Occurrence Levels of DBPs from Different Water Sources

Study	THM4 (μg/L)	HAA9 (μg/L)	Chloral hydrate (μg/L)	TOC (mg/L)	Bromide (mg/L)
[a]U.S. EPA ICR	39	20	1.7	2.4	0.04
[b]Priority DBP	31	34	1.0	5.8	0.12

[a]U.S. EPA ICR: McGuire et al., 2002.
[b]Priority DBP: Krasner et al., 2006.

Although the U.S. EPA ICR was conducted before the Krasner et al. (2006) DBP prioritization study, some of the priority DBPs were detected and measured in drinking water treatment plants under the ICR and a snapshot of their occurrence levels is shown in Table 1.5.

Table 1.4 Occurrence Levels of Some Priority DBPs in U.S. Drinking Water[a]

DBP	Minimum (μg/L)	Median (μg/L)	Maximum (μg/L)
2-Bromoacetamide	ND	ND	1.1
Bromonitromethane	ND	ND	0.3
Bromopicrin	ND	ND	5.0
Chloral hydrate	ND	1.0	16
Chloronitromethane	ND	ND	0.8
Chloropicrin	ND	0.2	2.0
Cyanogen chloride	ND	2.5	8.4
Dibromoacetonitrile	ND	0.2	2.0
Dichloroacetaldehyde	ND	1.0	14
Dichloroacetonitrile	ND	1.0	12

ND: Not detected.
[a]Krasner et al., 2006.

Table 1.5 Occurrence Levels of Some Priority DBPs from the U.S. EPA ICR[a]

DBP	Number of plants	Conc. range (μg/L)	90th percentile (μg/L)
Chloral hydrate	473	< 0.5 – 46	7.4
Dibromoacetic acid	11251	< 0.5 – 35	3.5
Dibromoacetonitrile	428	< 0.5 – 24	2.3
Dichloroacetonitrile	429	< 0.5 – 24.6	4.4
Trichloroacetic acid	11251	< 0.5 – 175	26

[a]McGuire et al., 2002.

The occurrence levels of priority DBPs as shown in Table 1.5 represent a very small fraction of DBP levels that consumers are actually exposed to via drinking treated water. A great deal of work still has to be done to characterize the unknown fraction of DBPs (Hua & Reckhow 2007; Krasner et al., 2006; Weinberg 1999) that might possibly affect human health including the human endocrine system when acting alone or in complex mixtures with other compounds.

1.1.7 Health Effects Associated with DBPs Found in Drinking Water

Since the discovery of DBPs over the last 35 years, a body of scientific research has evolved that is suggestive of the potential health effects associated with their presence in

drinking water. Some toxicological studies using laboratory animals have shown that DBPs such as trichloroacetic acid, dibromoacetic acid, dichloroacetic acid, bromodichloromethane, and chloral hydrate at elevated concentrations caused spermatoxicity (Linder et al., 1994; Klinefelter et al., 1995; Katz et al., 1981; Bhat et al., 1991). Orally administered dichloroacetic acid at elevated concentrations caused growth retardation, fetal death and malformation in developing rats (Smith et al., 1992) while developmental toxicity in rats have been reported due to trichloroacetic acid exposure (Smith et al., 1989). *In vivo* studies showed that 3-chloro-4-(dichloromethyl)-5-hydroxyl-2(5H)-furanone (MX) is a genotoxic compound that induced DNA damage in the brain and caused cancer at multiple sites in laboratory animals (McDonald & Komulainen 2005; Verschaeve et al., 2006; Komulainen et al., 1997), while results from another showed that iodoacetic acid caused developmental abnormalities and induced neural tube closure defects in mouse embryos (Hunter et al., 1996).

Human exposure to DBPs in drinking water does not occur from single compounds but combinations of them. An *in vivo* study showed that mixtures of bromochloroacetic, dichloroacetic and dibromoacetic acid at elevated concentrations caused adverse reproductive and developmental effects in rat embryo culture (Andrews et al., 2004) while another reported that combinations of THM4 and HAA5 induced pregnancy loss in rats (Narotsky et al., 2011). The effect of the DBP mixture in the Andrews et al. (2004) study had approximately the same activity as the single compounds. Such an observation showed that the DBPs probably acted through the same mode of action. The same observation was made from the Narotsky et al. (2011) study where pregnancy loss was observed in F344 rats administered THM4 (920 μmol/kg) and HAA5 (1231 μmol/kg). As a mixture containing a

lesser concentration of HAA5 (615 μmol/kg) and THM (613 μmol/kg), the same effect was comparable to that of HAA5 (1231 μmol/kg).

The results from these animal studies have been complemented by those from various epidemiological studies which suggested that the presence of some species of trihalomethanes in drinking water caused spontaneous abortion, low birth weight, intrauterine growth retardation (IUGR), and still births (Dodds et al., 1999; Gallagher et al., 1998; Waller et al., 1998; Wright et al., 2003; Savitz et al., 1995; Kramer et al., 1992; Bove et al., 1995; Swan et al., 1992). Other epidemiological investigators associated long term exposure to DBPs in drinking water with increased risk of cancer (Chevrier et al., 2004; Cantor 1997; Koivusalo et al., 1997; Doyle et al., 1997; King & Marlett 1996). Most of these epidemiological studies have focused on DBP exposure through ingestion of drinking water; however, a study comparing exposure routes through ingestion of drinking water, inhalation/dermal absorption during showering, bathing and swimming in pools showed that the risk associated with bladder cancer is higher through dermal and inhalation route of exposure when compared to ingestion (Villanueva et al., 2007).

1.1.8 Limitations of Results from *In Vivo* and Epidemiological Studies

Despite the potential health effects associated with DBPs, the limitations associated with toxicological and epidemiological studies cannot be ignored. The concentration of DBPs which animals are normally exposed to during toxicological studies does not represent the trace levels to which humans are exposed. Exposure of animals to DBPs during toxicological studies is mostly done by gavages whereas humans are exposed to these compounds via ingestion, dermal and inhalation routes while swimming, showering and bathing (Villanueva et al., 2007; Beech et al., 1980; McKone 1993). Based on these differences in routes of

exposure and concentration of DBPs used in toxicological animal studies, the health effects associated with these compounds should be interpreted with caution especially when applied to humans.

Another limitation associated with epidemiological studies is in the use of routinely collected measurements of THMs leaving the treatment plant as an exposure index (Kramer et al., 1992; Bove et al., 1995; Gallagher et al., 1998; Dodds et al., 1999). There is no evidence to suggest that THMs represent all DBPs in drinking water. This approach fails to account for changes in concentrations as the water travels to the consumer's tap or consumption from other sources such as the workplace or bottled water. Such routinely collected THM concentrations may not adequately reflect what individuals in epidemiological studies have actually been exposed to because DBP concentration varies with temperature, pH, disinfectant concentration, reaction time, and NOM concentration (U.S. EPA, 2005).

1.1.9 Mechanisms through which DBPs in Drinking Water can Cause Adverse Health Effects

The mechanisms through which DBPs cause adverse health effects have not been well investigated but some investigators have suggested oxidative stress, genotoxicity/mutagenicity, and folate metabolism as possibilities (Kim et al., 2005; Scholl et al., 2001; Karowicz-Bilinska et al., 2002; Geter et al., 2005; Plewa et al., 2002; Plewa et al., 2007). Oxidative stress in the body generates reactive oxygen species (ROS) that reacts with DNA to cause damage (Hracsko et al., 2008) and malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OH-dG) are examples of biomarkers that be measured as indicators of this stress. (Thompson et al., 1999; Drury et al., 1997). The levels of these

biomarkers in the urine of 261 pregnant women collected during their first stage of labor were determined by using an *in vitro* enzyme- linked immunosorbent (ELISA) kit and high performance liquid chromatography (HPLC) during an epidemiological study (Kim et al., 2005). Results from the Kim et al. (2005) study that showed reduced neonatal birth weight after adjusting for confounding factors have been complemented by others that showed correlations between increased levels of 8-OH-dG and MDA with Intrauterine growth restriction (IUGR) and low birth weight (Prabhu et al., 2010; Scholl & Stein 2001). The exact mechanism for these adverse reproductive outcomes has not been well defined but it has been suggested that a contributing factor includes exposure to chemicals in drinking water (Kim et al., 2005).

Genotoxicity refers to DNA damage that includes strand breaks and formation of adducts that can be caused by different mechanisms such as increased oxidative stress, incomplete DNA excision repair and increased intracellular calcium (Halliwell & Aruoma 1991). An *in vitro* study using mammalian cells has demonstrated that certain DBPs such as haloacetic acids are genotoxic (Plewa et al., 2002), complemented by another study showing unregulated haloacetonitriles and halonitromethanes having the same property (Plewa et al., 2007). Results from the Plewa et al. (2002) study also highlighted that the bromine-containing haloacetic acids were more genotoxic than the chlorinated ones. Another *in vitro* study using the bacterium *Salmonella typhimurium* also showed the haloacetic acids as genotoxic DBPs; however, the results were not consistent in terms of rank order potency when compared to those obtained using the mammalian cells in the Plewa et al. (2002) study. Results from these *in vivo* studies show the importance and need to address the presence of

the unregulated DBPs in drinking water in addition to the regulated ones that have specified MCLs under the U.S. EPA DBP rules.

Folate metabolism is a mechanism through which DBPs have been suggested to cause adverse health effect such as cancer and studies have showed that aberrant crypt foci (ACF) are precursor lesions in the development of colon cancer (Konstantakos et al., 1996; Siu et al., 1997). Folate is one form of vitamin B that is involved in synthesis, repair and functioning of DNA as well as cell production and maintenance (Kamen 1997) and it has an increased need in pregnant/lactating women, alcohol abusers, and individuals with liver disease. A deficiency in this vitamin can cause DNA strand breaks, chromosomal damage and cancer due to misincorporation of nucleobases during DNA synthesis (Reidy 1988). *In vivo* studies involving laboratory animals exposed to elevated concentrations of brominated THMs in drinking water showed induced formation of ACF (DeAngelo et al., 2002; Geter et al., 2004). This observation was also made in another study that exposed F344/N rats to tribromomethane at a concentration of 500 mg/L in drinking water, and significant differences were recorded when the exposure was combined with a no folate diet (Geter et al., 2005). Apart from being carcinogenic, DBPs can also cause detrimental health effects when acting in combination with other factors such as diet as observed in the Geter et al. (2005) study.

1.1.10 Supporting Evidence of the Effect of Endocrine Disruptors in Wildlife and Humans

Some chemicals have the ability to affect the endocrine system and are referred to as endocrine disrupting compounds (EDCs). EDCs such as phytoestrogens exist naturally in plants while others like pesticides, herbicides, and pharmaceuticals are products of human

industry. According to the U.S. EPA, an endocrine disrupting compound is "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (U.S. EPA, 1997).

A significant number of studies have shown that certain chemicals released into the environment have the ability to alter the function of the endocrine system in exposed species (Purdom et al., 1994; Barnhoorn et al., 2004; Jobling & Tyler 2003; Tyler et al., 1998; Panter et al., 1998; Balch et al., 2004; Brion et al., 2004; Kramer et al., 1998; Imai et al., 2005). Results from one study clearly demonstrated that lowered testosterone and increased 17-β-estradiol (E2) levels observed in alligators residing in Lake Apopka in Florida were associated with exposure to the pesticide dicofol and herbicide dichlorodiphenyltrichloroethane (DDT) (Guillette et al., 1994; Falconer 2006; Juberg 2000). Tributylin (a constituent in anti-fouling paints) used on boats for preventing the growth of barnacles on ships caused infertility, imposex and extinction of some mollusk species (Matthiessen 2003; Barlow et al., 1999; Ellis & Pattisina 1990) and its use in the U.S. has been restricted under the Anti-fouling System Control Act of 2007 due to its adverse environmental impact (U.S. EPA, 2007).

The most compelling evidence of endocrine disruption due to environmental exposure has been documented in studies involving aquatic organisms since they reside in surface waters that act as main sinks for EDCs of anthropogenic origin. Some reports from these studies have shown that fish breeding downstream of wastewater treatment plants exhibited abnormal reproductive systems, altered growth, and reduced spawning success most likely due to the presence of EDCs (Jobling et al., 1998; Vajda et al., 2008; Renner 2009). Other

studies showed masculinization and inhibition of spermatogenesis in fish exposed to Kraft/pulp mill and sewage treatment plant (STP) effluent containing EDCs (Bortone & Davis 1994; Larsonn & Forlin 2002; Parks et al., 2001; Diniz et al., 2005). Vitellogenin is an egg yolk protein precursor that would normally be found in female fish due to the presence of estrogen; however, it has been well documented that the presence of natural and synthetic hormones in sewage effluent stimulates the endocrine system of this protein in different species of male fish and causes feminization (An et al., 2008; Liney et al., 2006; Nakari 2004; Gross-Sorokin et al., 2006).

The degree to which humans are affected by the EDCs is unknown. Studies have shown observations of uncommon vaginal adenocarcinoma and reproductive abnormalities in individuals whose mothers were treated with the synthetic estrogen diethylstilbestrol (DES) (Herbst 1981; Sharpe & Shakkebaek 1993; Mittendorf 1995). Reports have also associated EDCs with decreased sperm counts, increased incidences of certain types of cancer, and type II diabetes (Hodgson et al., 2004; Rozati et al., 2000; Lang et al., 2008; Glass & Hoover 1990). Despite these observations, some investigators have concluded that there is no evidence of risk from environmental sources of endocrine disrupting chemicals in humans (Kavlock et al., 1996; Solomon et al., 1998; Waddell 1998; Daston et al., 1997; Juberg et al., 2000; Cargouet et al., 2007).

1.1.11 Pathways for EDCs into Drinking Water Sources

EDCs of anthropogenic origin have multiple uses and this generates multiple pathways through which they can enter surface and groundwater as shown in Figure 1.1.

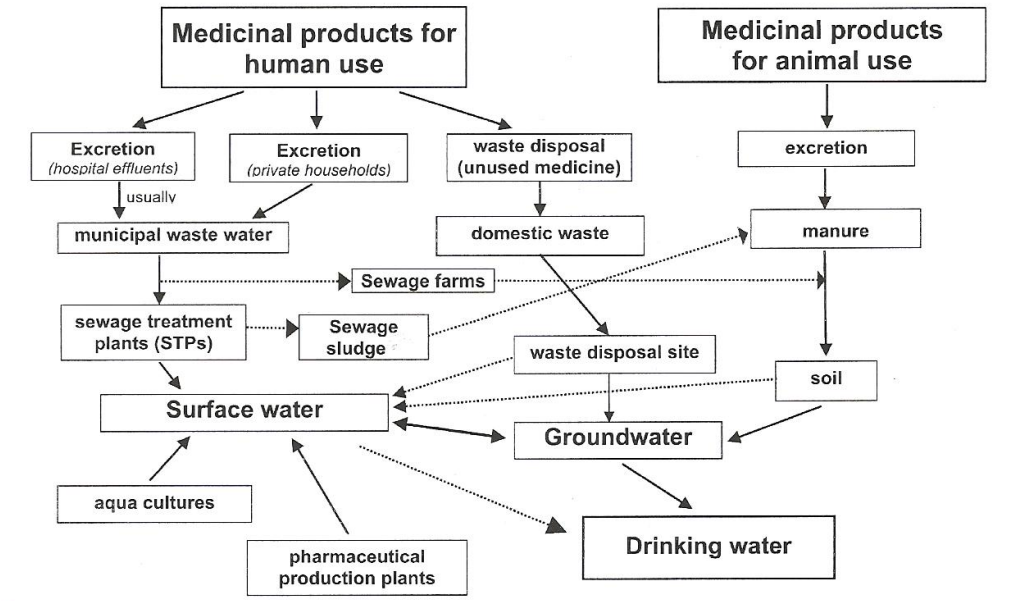


Figure 1.1: Pathways for EDCs in Drinking Water Sources (Herberer, 2002)

Excretion of hormones from living organisms represents one of these pathways and E2 is excreted at a daily rate between 2.3 and 3.5 μg per day by menstruating/menopausal women (Hu et al., 2003; Johnson et al., 2000). This rate is even higher for pregnant women with reported levels between 259 and 400 μg per day (Racz & Goel 2009; Hu et al., 2003). E2 found in urine is generally lower in men with reported levels of 1.6 μg per day (Johnson et al., 2000) while its metabolites estrone (E1) and estriol (E3) are excreted by menstruating/menopausal women at rates between 1 and 8 μg per day (Johnson et al., 2000). The excretion rates of E2 in livestock urine are generally higher than humans with estimated levels as high as 500 and 163000 μg per day for non-pregnant and pregnant dairy cattle, respectively (Hanselman et al., 2003). These hormones are not completely metabolized in humans and animals before they are released into municipal waste water and can be

reconverted to parent compounds by bacteria in sewage treatment plants or aquatic environment (Racz & Goel 2009; Hu et al., 2003).

Improper disposal of unused pharmaceutical drugs is another pathway through which EDCs enter surface waters. Unwanted prescription drugs are often flushed down the toilet or disposed with household waste as trash. Such improper disposal methods cause drugs to end up in landfills and waste water treatment plants (WWTPs) (Heberer 2002). Pharmaceuticals such as atenolol, gemifibrozil, erythromycin, and ranitidine have been detected in streams and drinking water (Kolpin et al., 2002). A public health concern associated with detection of these compounds arises from an *in vitro* study that showed their estrogenic activity (Isidori et al., 2009) while another study reported positive estrogenic responses from pharmaceuticals such as paracetamol, fenofibrate, cimetidine, and furosemide (Fent et al., 2006).

An issue associated with estrogen being discharged into municipal wastewater and landfills is that they end up in surface water since WWTPs are not designed to remove them completely. The activated sludge process in wastewater treatment removes the bulk of the organic compounds that enter the plant and various efficiency removal rates have been reported for the steroids. One study showed removal rates of 61, 87, 95 and 85 percent for E1, E2, E3, and 17-α-ethynylestradiol (EE2) respectively (Baronti et al., 2000) while another reported 74, 88 and 77 percent for E1, E2, and E3 (Johnson et al., 2000). Yet another study showed rates as high as 92 to 99.9 percent for E2 while E1 had a lower efficiency of removal of between 67 to 83 percent (Ternes et al., 1999). The endocrine system of aquatic organisms in surface water receiving effluent from WWTPs would be impacted by the presence of these incompletely removed estrogens that have been reported at levels as low as 1 ng/L with E1

usually having the highest concentration (Desbrow et al.,1998; Ternes et al., 1999; Belfroid et al., 1999).

Apart from incomplete removal, little is known about the possible transformation products produced during wastewater treatment processes. *In vitro* studies showed that chlorinated E2, bisphenol A (BPA), and 4-nonylphenol generated different transformation products with estrogenic activity. The estrogenic activity of chlorinated E2 solutions at 10, 30 and 60 minutes was similar but slightly lower than the unchlorinated parent compound (Hu et al., 2003) while 4-nonylphenol solution chlorinated for 10 minutes generated a reduced estrogenic response of 30 percent (Hu et al., 2002). BPA showed a different trend from E2 and 4-nonylphenol with its estrogenic activity slightly increased after an hour of chlorination (Alum et al., 2004). Results from these chlorinated EDC studies showed that increased chlorination time decreased the activity of the respective compounds. Such lengthy chlorination reaction time may not be practical for WWTPs but has been shown to decrease or completely remove the estrogenic activity of E2, BPA and NP after 24 hours (Lee et al., 2004).

1.1.12 Occurrence Levels of Steroid Hormones and EDCs in U.S. Streams and Drinking Water

Some studies have identified the occurrence level of suspected or known EDCs in surface and groundwater in the U.S. (Benotti et al., 2009; Focazio et al., 2008; Barnes et al., 2008). A study conducted by the U.S. Geological Survey (USGS) measured concentrations of 95 organic wastewater contaminants (OWCs) from 139 streams (Kolpin et al., 2002). The levels of OWCs reported in this study should not be taken as a total representative of those found in the U.S. since the streams used in this study were susceptible to contamination from

urbanization and livestock production. Levels of steroid hormones and some EDCs found in drinking water sources from this study are summarized in Table 1.6.

Table 1.6 Occurrence Levels of EDCs found in U.S. streams (Kolpin et al., 2002)

Compound	Median (μg/L)	Max (μg/L)	Frequency (%)
17-α-estradiol	0.03	0.074	5.7
17-α-ethynylestradiol	0.073	0.831	15.7
17-β-estradiol	0.009	0.093	10
4-nonylphenol	0.8	40	50.6
4-octylphenol diethoxylate	1.0	9.0	36.5
Bisphenol A	0.14	12	41.2
Erythromycin	0.1	1.7	21.5
Estriol	0.019	0.051	21.4
Estrone	0.027	0.112	7.1
Gemfibrozil	0.048	0.79	3.6
Progesterone	0.11	0.199	4.3
Ranitidine	0.01	0.01	1.2
Stigmastanol	2.0	4.0	5.6

Among the identified EDCs, natural and synthetic hormones derived from human and animal release are more estrogenic than others normally found at higher concentrations in surface and groundwater as shown in Figure 1.2.

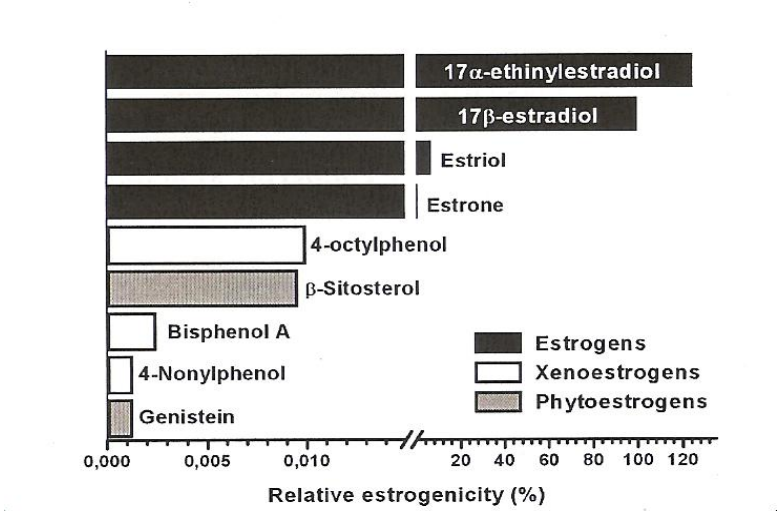


Figure 1.2: Relative Estrogenic Activities of some EDCs found in Drinking Water Sources

1.1.13 Source Water Quality Protection

Human waste, pesticides, and improperly disposed chemicals can contaminate drinking water sources. In order to protect public health, the Safe Drinking Water Act (SDWA) was established in 1974 and it regulates the nation's drinking water except private wells that serve less than 25 individuals. The Act also authorizes the U.S. EPA to set standards for drinking water while working with states and water systems to ensure compliance (U.S. EPA, 1974).

One of the tools used by the SDWA is the National Primary Drinking Water Regulations (NPDWRs) that limit the levels of contaminants found in drinking water and has well established enforceable standards or MCLs for microorganisms, disinfectants, DBPs, radionuclides, and organic/inorganic chemicals (U.S. EPA, 2009a). Unlike the MCL, Maximum Contaminant Level Goals (MCLGs) are not enforceable standards but are set at levels for which no adverse health effects are known to occur in humans. A regulated DBP such as chlorite has a MCLG/MCL in drinking water of 0.8 and 1.0 mg/L, respectively while that for bromate is 0 and 0.010 mg/L. THM4 do not have a collective MCLG but some individual species such as chloroform, dibromochloromethane and bromoform have one of 0.07, 0.06, and 0 mg/L, respectively, in drinking water. Haloacetic acid species such as trichloroacetic, monochloroacetic acid, and dichloroacetic acid also have a MCLG of 0.02, 0.07, and 0 mg/L, respectively, in drinking water (U.S. EPA, 2006b).

Apart from the NPDWRs, the U.S. EPA also uses the National Secondary Drinking Water Regulation (NSDWRs) to address 15 contaminants. Standards set under the NSDWRs are known as the Secondary Maximum Contaminant Levels (SMCLs) and are not enforceable by the agency. SMCLs were established as a guideline to assist public water

utilities in managing their drinking water for aesthetic considerations but exceeding their values does not pose any health risk to humans. Public water utilities with exceeded values usually have cloudy water with an odor and this discourages consumers' consumption even though it is still safe (U.S. EPA, 1991).

Unregulated contaminants that may require drinking water regulation in the future are also identified by the SDWA and placed on the Contaminant Candidate List (CCL). There are currently 116 chemicals and microbial contaminants listed on the Contaminant Candidate List 3 (CCL3) and the U.S. EPA decides which ones would be regulated based on research and occurrence data. Unregulated estrogenic hormones such as E1, E2, E3, EE2 with confirmed adverse health effects on aquatic organisms are listed on the CCL including DBPs such as N-nitrosodimethylamine (NDMA), N-nitroso-di-n-propylamine (NDPA), N-nitrosodiphenylamine, N-nitrosopyrrolidine (NPYR) and N-nitrosodiethylamine (NDEA) (U.S. EPA, 2009b).

The Drinking Water Strategy (DWS) is another approach that was developed recently in 2010 by the U.S. EPA to protect the nation's drinking water and its goals are to address contaminants found in drinking water as a group, foster development of new drinking water technologies, apply the authority of multiple statutes to drinking water, and provide a network between states so that data on drinking water can be shared (U.S. EPA, 2010a). The agency hopes that these goals provide more public health protection and assist communities in identifying cost effective treatment technologies to provide water to consumers at a reasonable cost.

1.1.14 The Endocrine System – A Master Regulator of Development

The endocrine system, also known as the hormone system, consists of glands, hormones, and receptors (Toppari 2008; U.S. EPA, 1998c). The glands are located throughout the body and are responsible for synthesizing and secreting hormones. Hormones act as chemical messengers and are released into the bloodstream and their goal is to find compatible receptors in the various target tissues and organs. More than 50 hormones with different functions have been identified in the endocrine system of humans and other living organisms. The antidiuretic hormone is an example of a hormone produced by the pituitary gland that helps to stimulate water reabsorption by the kidney tubules. Others include adrenaline and noradrenaline produced by the adrenal glands to help the body react under stressful conditions. The endocrine system found in nearly all animals basically regulates a wide range of biological processes like metabolism, growth, reproduction, and development of the brain (U.S. EPA, 1998c). Disturbances of this system that may involve overactive and underactive hormone secretion can cause harmful effects in the body (Nilsson 2000; Toppari 2008; Lee et al., 2010; Henley & Korach 2006).

1.1.15 Genomic Mode of Action of Steroid Hormones

Steroids represent one of the major classes of hormones synthesized from cholesterol and consist of the sex hormones known as estrogen and androgen (U.S. EPA, 1998c). Estrogens are steroids biosynthesized in the ovaries, adrenal gland and testes. The steroid is responsible for female sexual characteristics and the main one found in non-pregnant females is E2. Androgens are responsible for male sexual characteristics and also play a crucial role in the development of their reproductive organs. Testosterone is an example of an androgen mainly secreted by the Leydig cells in the male testes (Bovee & Pikkemaat 2009).

Steroid hormones are involved in the genomic and non-genomic pathway in order to generate a biological response in the body (Sharma 1999; Beato et al., 1996). The former begins when the steroids are released into the bloodstream, and their goal is to find compatible receptors in tissues and organs. Inactive steroid receptors are usually located in the cytoplasm of a cell and have complexes called heat shock proteins attached to them. When a steroid hormone diffuses across the cell membrane and gets into the cytoplasm of a cell, it binds to its compatible receptor. This action in addition to phosphorylation causes the receptor to become activated, thereby causing the dissociation of the heat shock proteins. The complex formed at this point (consisting of two receptors having a molecule of hormone) crosses into the nucleus of the cell and binds to hormone responsive elements on a gene. This action causes the gene to be activated and undergoes transcription to form messenger ribonucleic acid (mRNA) that eventually gets transported into the cytoplasm. Ribosomes in the cytoplasm causes translation of mRNA to form protein and this final product can be a peptide hormone, growth factor or enzyme that plays major functions as part of the endocrine system (U.S. EPA, 1998c).

The protein produced in the genomic pathway as shown in Figure 1.3 can be E2, which when released into surface water, has been shown to disrupt the endocrine system of fish by causing intersexuality (Jobling et al., 1998). In order to characterize and rank the endocrine disrupting potential of chemicals found in surface water and DBPs in drinking water, it is necessary to employ *in vitro* assays that measure products taken as indicators of the amount of protein produced in the genomic pathway.

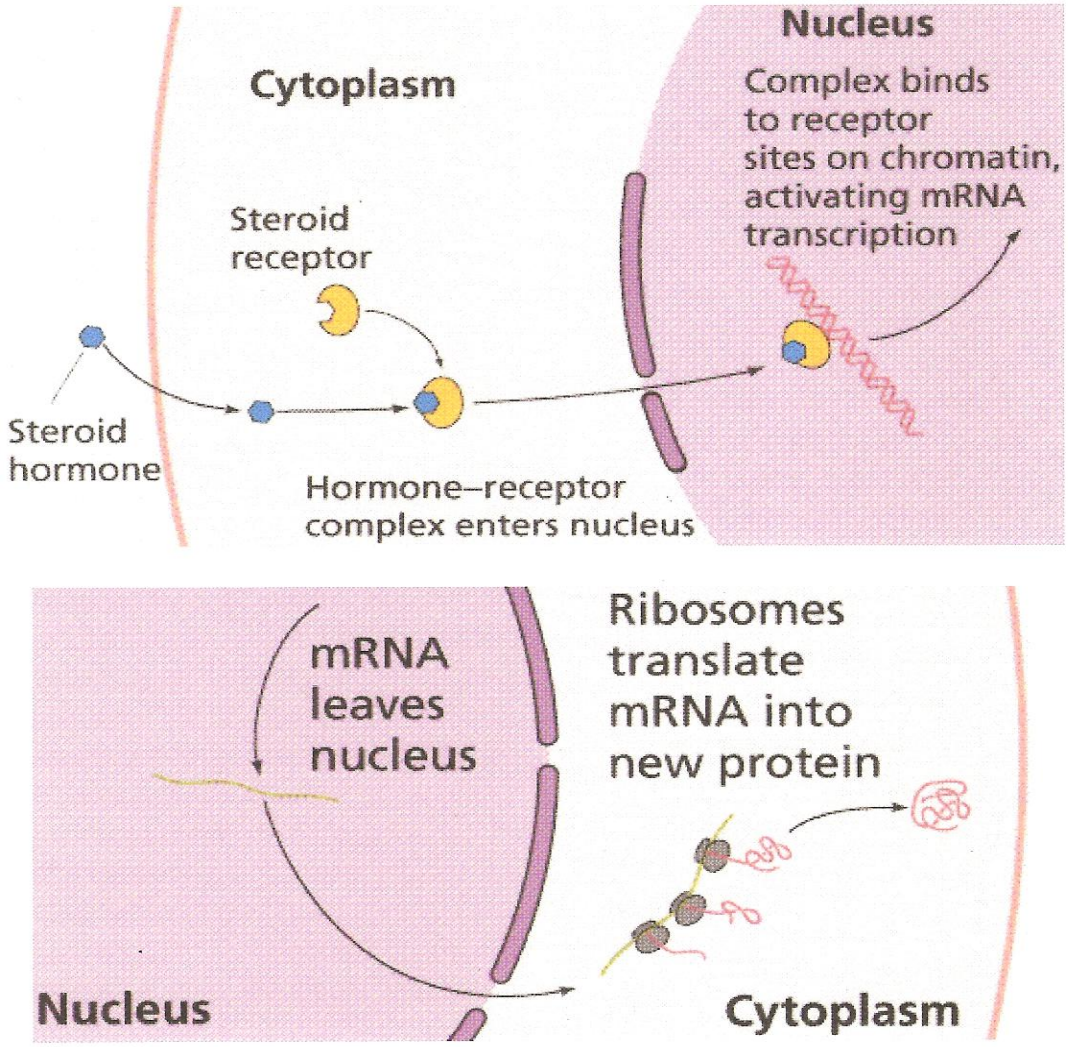


Figure 1.3: Genomic Mode of Action of the Steroid Hormone (Purves, 1998)

1.1.16 Mechanisms by which EDCs could Influence the Endocrine System

Different pathways by which EDCs could influence the endocrine system while acting through the genomic mode of action associated with steroid hormones have been proposed (Hewitt & Korach 2002; Toppari 2008; Henley & Korach 2006; Lee et al., 2010; Hall et al., 2001; Deroo & Korach 2006; U.S. EPA, 2010b). EDCs can act in place of a steroid hormone that would normally bind to a compatible receptor. Such mimicking actions of EDCs can cause generation of a biological response to occur at an inappropriate time or

one that would be much stronger than that of the natural hormone. An example of such a situation may involve generation of growth factors at inappropriate time, thereby leading to increased muscle mass. A second proposed mechanism through which EDCs could influence the endocrine system is by acting as anti-estrogenic or anti-androgenic compounds preventing the normal binding of the natural steroid hormone to its receptor. An action like this modifies the secretion, synthesis, binding action, transport, or elimination of the natural steroid hormone. Anti-estrogenic or anti-androgenic activities of EDCs could cause abnormal development due to improper synthesis of growth factors by the endocrine system (U.S. EPA, 2010a). A third proposed mechanism involves interaction with the enzymes that are responsible for synthesizing the steroid hormones. Aromatase (a cytochrome P450 enzyme) converts testosterone to E2. EDCs can interfere with the normal functioning of this enzyme, thereby causing overproduction or underproduction of E2.

1.1.17 Predicting Estrogenic Activity of Exogenous Chemicals

There are significant challenges in using structures to determine if chemicals are capable of disrupting the endocrine system. Structures of steroid hormones and other chemicals that have been shown to have a relative binding affinity to the estrogen receptor are shown in Figure 1.4. Structural similarities between steroids and compounds with confirmed estrogenic activity include the presence of two rings (one of them usually phenolic) separated by two carbons (Fang et al., 2001).The presence of a phenolic group on a molecule is normally an indication that it would most likely bind to the estrogen receptor (McLachlan 1985; Sumpter & Routledge 1997). A compound such as 4-n-nonylphenol that possesses the phenolic ring but lacks a second ring possesses a lower relative binding affinity to the estrogen receptor when compared to E2 and some other EDCs.

The relative binding affinity of steroid hormones and other chemicals to the estrogen receptor is strongly enhanced by the presence of hydroxyl groups on the A and D rings (Bohl et al., 1987). The hydroxyl group of the phenolic A-ring is involved in binding with specific amino acids on the estrogen receptor (Fang et al., 2001). Modification of the steroid hormone structure and that of other chemicals by the addition of other functional groups reduces their relative binding affinity to the estrogen receptor, thereby causing reduced estrogenic activity. The addition of a hydroxyl group at position C-2, C-4, and C-16 of steroid hormones has also been shown to cause reduced estrogenic activity and this criterion explains why estriol has a lower activity when compared to estrone (Tanaka et al., 2001).

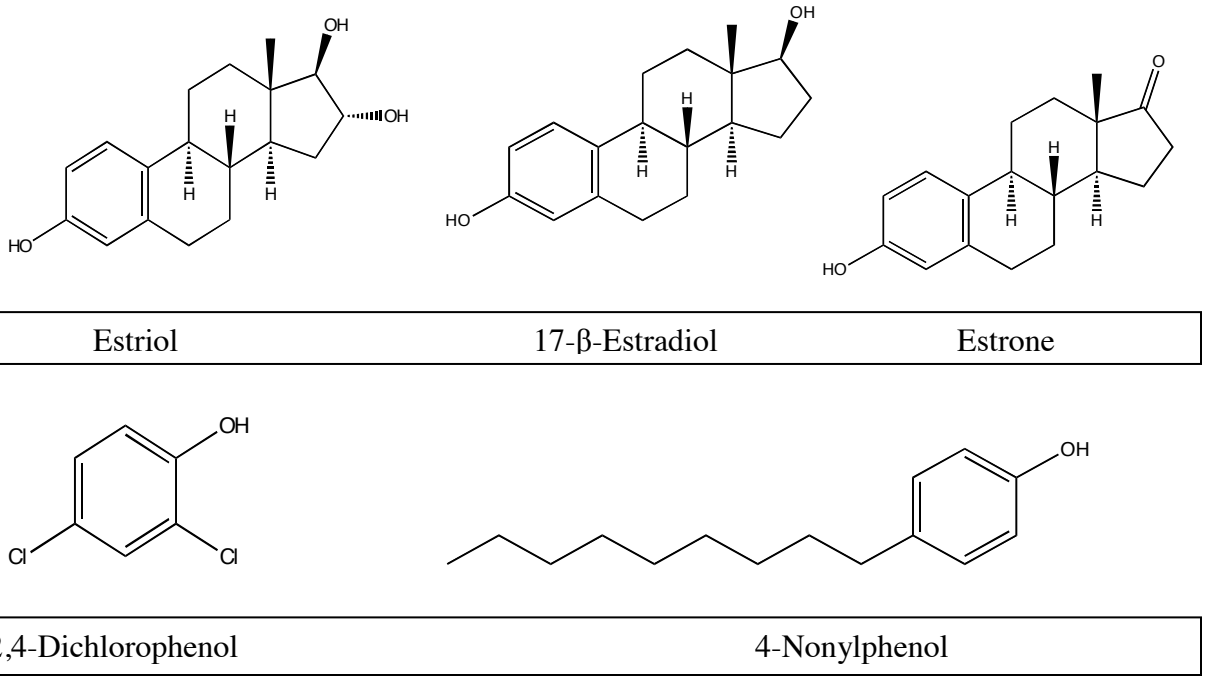
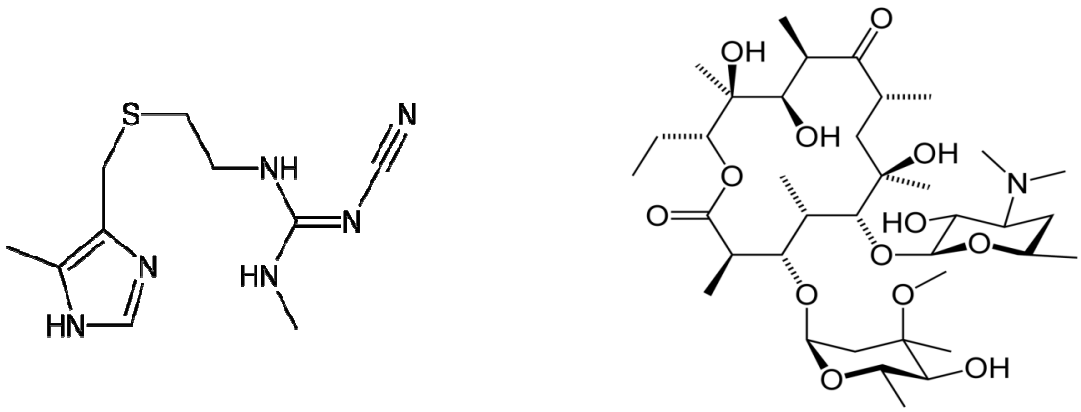


Figure 1.4: Structures of Hormones and other Chemicals with Confirmed Estrogenic Activity

While there appears to be some generalized common structural features among EDCs found in surface waters, the criteria described above cannot be applied to all chemicals in order to determine their estrogenic activity. As an example of this challenge, an *in vitro* study

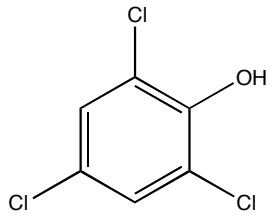
using the yeast estrogen screen (YES) assay showed that the pharmaceutically active compounds erythromycin and cimetidine induced estrogenic activity (Isidori et al., 2009) while an *in vivo* study suggested that the drinking water DBP dibromoacetic acid may also have such activity (Goldman & Murr 2003).

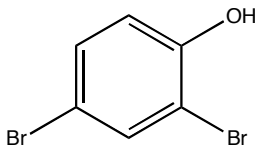
As shown in Figure 1.5, the pharmaceuticals from the Isidori et al. (2009) study do not have the structural features described previously. Results from *in vitro* studies showed that the phenolic compounds shown in Figure 1.6 induced no estrogenic activity despite having structural similarities to 2,4-dichlorophenol and 2-chlorophenol (Nishihara et al., 2000; Olsen et al., 2002). Disinfection can cause transformation of chemicals into products that may be less estrogenic (Hu et al., 2003; Alum et al., 2004; Lee et al., 2004) but the inability to identify the chemical structure of all transformation products may become an issue when trying to predict estrogenic activity in drinking water.



Cimetidine	Erythromycin

Figure 1.5: Structures of Pharmaceuticals and DBPs with Suspected Estrogenic Activity





2,4,6-Trichlorophenol	2,4-Dibromophenol

Figure 1.6 Structures of Phenolic Compounds Lacking Estrogenic Activity

1.1.18 Response to the Presence of EDCs found in the Environment

In 1996, the United States Congress responded to societal concerns by making amendments to the Food Quality Protection Act (FQPA) and the Safe Drinking Water Act (SDWA). These amendments gave the U.S. EPA authority to develop a screening and testing program by using validated test systems and scientific information to determine if pesticide chemicals and substances found in drinking water had the potential to cause effects that would normally be produced by human hormones. Based on these amendments, the agency also had the authority to screen other chemicals that could also affect other pathways of the endocrine system. In October 1996, the Endocrine Disruptor Screening and Testing Advisory (EDSTAC) was assembled to provide advice to the U.S. EPA on how to design a screening and testing program for endocrine disruptors. Recommendations from the EDSTAC required the U.S. EPA to evaluate both human and ecological (wildlife) effects, examine effects related to estrogen, androgen and thyroid hormone processes as well as testing both individual substances and common mixtures (U.S. EPA, 1998c). Among the EDSTAC recommendations, it was suggested that DBPs be included in the mixtures evaluated for endocrine disrupting activity based on suspected interference with the carefully regulated hormonal messenger system, a finding that has been confirmed by various studies (Brittebo

et al., 1987; Goldman & Murr 2003; Linder et al., 1994; Klinefelter et al., 1995; Katz et al., 1981; Bhat et al., 1991). Based on these recommendations, the U.S. EPA proposed the endocrine disruptor screening program (EDSP) in December 1998.

1.1.19 Assays Employed for Measurement of Estrogenic Activity of EDCs

Although chemical methods can provide occurrence data on EDCs in water, an evaluation of endocrine activity requires the use of biological assays. Inexpensive and "quick-to-perform" assays such as estrogen/androgen reporters included under the U.S. EPA Tier 1 screening are meant to serve as a screening tool to determine if compounds or mixtures are estrogenic or not. Results from initial screening are then used to determine which compounds would be subjected to Tier 2 Testing that employs *in vivo* assays such as a two-generation mammalian reproduction and the mysid shrimp reproduction test (U.S. EPA, 1998c). Tier 2 testing is typically more expensive and time consuming than those assays employed under Tier 1 screening, but results using animal cells are a better representation of how humans might be affected by EDCs since they address absorption, distribution, metabolism and excretion (ADME).

The *in vitro* assays employ some of the pathways activated by the natural estrogens in the body and fall under four categories that include estrogen receptor binding, reporter gene, gene expression, and cell proliferation assays (Zacharewski 1997). Each category has certain advantages and disadvantages over the other based on limit of detection of estrogenic activity, cytotoxicty of cell lines due to presence of EDCs, ease of use, cost and reproducibility (Leusch et al., 2006; Zacharewski 1997). In particular, they do not pinpoint the chemicals responsible for generating the estrogenic activity in a sample containing a complex mixture of chemicals and so there is sometimes the need to use chemical analysis

simultaneously (Jugan et al., 2009; Liu et al., 2005). These assays do not address metabolism of parent compounds that occurs in living organisms so it is not logical to conclude that a negative estrogenic response applies to the activity of their metabolites unless they are analyzed as well.

Estrogen Receptor Binding Assays

The estrogen receptor binding assay is a relatively quick method that is used to determine if a compound has an affinity for the estrogen receptor. This assay helps to measures the fraction of a labeled estradiol that is displaced when in the presence of an unlabeled compound (NIEHS, 2003). If the compound has an affinity for the estrogen receptor, the fraction of the labeled estradiol would basically decrease as the concentration of the suspected EDC increases. The receptor binding assays represent and focus on the initial step of the genomic mode of action of steroid hormones which involves binding of a ligand to the estrogen receptor (Danzo 1997). In living organisms, the ligand-estrogen receptor complex eventually leads to the transcription of genes that causes generation of proteins that perform numerous functions. Despite the advantage of being relatively quick, this class of *in vitro* assay has its disadvantages in that it only determines if a suspected EDC has an affinity for the estrogen receptor and does not address nor simulate the cascade of events that ultimately lead to the generation of proteins. Uncompetitive displacement of the labeled estrogen is also another limitation especially when the concentration of the EDC is very high (Zacharewski 1997).

The limit of detection (LOD) for an *in vitro* assay is estimated as the lowest concentration that that leads to a significant increase in the end product being measured when compared to the activity of the negative control (Jugan et al., 2009; Sumpter & Routledge

1996) and it's established by assaying a serially diluted concentration of E2 that yields a full dose-response curve. E2 is normally used to establish this limit since it is the most estrogenic natural steroid hormone. The estrogen receptor binding assay can be used to determine the estrogenic activity of environmental samples but a limit of detection of 272 ng/L also serves as a drawback when compared to the other *in vitro* assays that measure estrogenic activity. Such an insensitive limit of detection can cause increased risk of false negatives so this class of assay should be complemented with the sensitive ones (Murk et al., 2002).

Fluorescence- Polarization Estrogen Receptor (FP-ER) assay

The FP-ER assay is an estrogen receptor binding assay that measures the fraction of a fluormone that is displaced from an estrogen receptor (Parker et al., 2000). The suspected EDC or sample is incubated with the estrogen receptor- fluormone complex and those that have an affinity for the estrogen receptor receptor displace a fraction of the fluormone. The amount displaced is determined by polarization which involves measurement by a fluorescence polarization instrument. The polarization of the new complex consisting of the sample or suspected EDC should be less than that of estrogen receptor- fluormone complex after displacement has occurred.

The Standard ER Binding Assay

The Standard ER binding assay is also based on competition for the estrogen receptor by samples and suspected EDCs. Purified estrogen receptor is incubated with radiolabelled E2 in buffer and a dose range of compounds or samples before incubation. After 18 to 24 hours of incubation, the unbound ligand is removed by dextran-coated charcoal or hydroxyapatite. The amount of radiolabelled E2 that is still attached to the estrogen receptor

is then measured by a scintillation counter and its percentage is plotted against the logarithm of the increasing molar concentration of the compound (NIEHS, 2003).

Reporter Gene Assays

Reporter gene assays have an advantage over the receptor binding assays in the sense that they simulate the cascade of events that ultimately leads to production of proteins in living organisms. Luciferase and β-galactosidase are enzymes produced respectively by the recombinant yeast and mammalian cell report gene assays. These enzymes react with substrates to generate a product that can be measured as an indication of the proteins that would be generated by steroid hormones employing the genomic mode of action. (Wilson et al., 2004; Sumpter & Routledge 1996).

Recombinant Yeast Assays

Recombinant yeast assays have been used extensively to determine the estrogenic activity of EDCs and environmental water samples (Beck et al., 2006; Wu et al., 2009; Rutishauser et al., 2004; Leusch et al., 2010; Cespedes et al., 2004; Garcia-Reyero et al., 2001; Chen et al., 2007). Assays in this class employ yeast strains that have the human estrogen receptor transfected into it, thereby preventing unintended interaction of the suspected EDC or environmental sample with other receptors that are usually present in mammalian cell lines (Sumpter & Routledge 1996). Compounds that show positive estrogenic activity when analyzed with recombinant yeast assays can be said to activate the estrogen receptor - mediated genomic pathway as shown in Figure 1.3.

False negative results have been suggested as a limitation for this class of assays due to the inability of the compound to cross the cell wall of the yeast strain employed (Legler et al., 2002; Nishihara et al., 2000; Zacharewski 1997). These assays do not address the non-

genomic pathway that might be a possible route for endocrine disruption and some compounds have been reported to creep between wells, thereby contaminating the wells containing non estrogenic compounds. The solvents in which the environmental samples are made also serve as a hindrance when using this assay. Solvents such as methyl tert-butyl ether have been shown to dissolve the plastic plates employed while using the assay, thereby making measurement of β-galactosidase activity unreliable (Bresford et al., 2000).

The most commonly used recombinant yeast assay is the yeast estrogen screen (YES) that employs yeast cells (*Saccharomyces cerevisae*) with the DNA sequences of the human estrogen receptor incorporated in its main chromosome (Sumpter & Routledge 1996). This assay can detect estrogenic activity at levels as low as 1.5 ng/L. As shown in Figure 1.7, the yeast cell has a plasmid containing a promoter, estrogen response elements (ERE) and a *Lac-Z* gene. When a compound crosses the cell wall of the yeast, it binds to the estrogen receptor and the complex formed binds to the ERE. This action allows the recruitment of transcription factors that enables the *Lac-Z* gene to undergo transcription and produce the enzyme (β-galactosidase) that is released into the assay medium. β-galactosidase metabolizes the chromogenic substrate (chlorophenol red-β-D-galactopyranoside) into galactose and chlorophenol red and the estrogenic activity of the suspected EDC or environmental sample is determined by measuring the formation of the red product (chlorophenol red) with a plate reader at an absorbance of 540 nm. Results obtained from chemical analysis of environmental water samples for estrogenic activity have been shown to be in good correlation with those obtained from using the YES (Leusch et al., 2010). Other forms of the yeast assays have also been employed to determine the estrogenic activity of chemicals/environmental samples and an example is the Yeast Two-hybrid assay (Nishihara

et al., 2000; Lee et al., 2004; Hu et al., 2003; Chen et al., 2007; Isidori et al., 2009). Noted variations from these assays include the yeast strain being used, number of estrogen response elements, duration of incubation of the sample, and use of o-nitrophenyl-β-galactosidase (o-NPG).

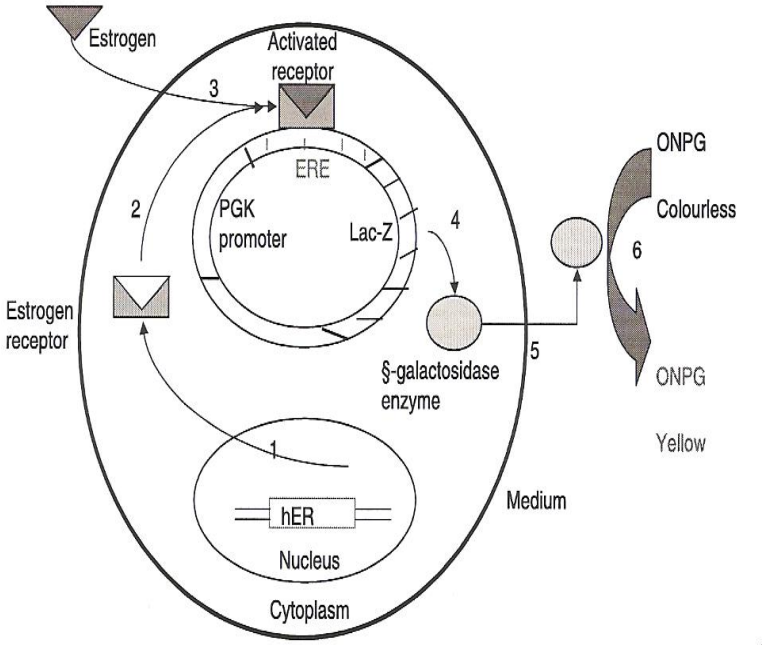


Figure 1.7 Schematic of the Molecular Basis for the YES Assay (Linden et al., 2007)

Recombinant Mammalian Cell Reporter Gene Assays

Recombinant mammalian cell reporter gene assays are typically more expensive and time intensive when compared to the yeast based assays but are more sensitive (Wilson et al., 2004; Legler et al., 1999; Balaguer et al., 1999; Leusch et al., 2010). These assays do have endogenous estrogen receptors that express both ERα and ERβ. Expression of both forms of the estrogen receptor would be a better representation of how EDCs could possibly affect the human endocrine system. Assays that fall under this category include the ER-mediated chemical-activated luciferase gene expression assay (ER-CALUX), MCF-7-ERE-

βGlob-Luc-Neo (MELN), and T47D-KBluc assays with a limit of detection of 0.1, 0.27 and 0.2 ng/L respectively (Leusch et al., 2010). A common feature among these assays is the presence of a plasmid containing a gene that has the ability to release luciferase when the estrogen receptor pathway is activated. The amount of luciferase produced is measured by a luminometer and taken as an indication of the estrogenic activity of the compound or sample. Chemical analysis of environmental samples for estrogenic activity involves the use of instrumentation such as High Pressure Liquid Chromatography with UV detector (HPLC-UV) and requires the known relative potency of each chemical being analyzed by instrumentation. In order to determine the contribution of a target analyte to the overall estrogenic activity of a sample, its concentration is determined by chemical analysis and multiplied by its relative potency in order to get an estradiol equivalents (EEQs) expressed in ng/L. The relative potency is calculated by dividing the EC_{50} of E2 by that of the analyte. For samples with more than one target analyte, their estrogenic activity contribution would be the sum of their individual EEQs (Beck et al., 2006; Rutishauser et al., 2004; Jugan et al., 2009). Recombinant mammalian cell reporter gene assays have been used for analysis of environmental water samples (Cargouet et al., 2007; Itoh et al., 2000) but a study showed that T47D-KBluc overestimated estrogenic activity when compared to that obtained from chemical analysis, while results using the MELN showed an underestimation (Leusch et al.,2010).

Gene Expression Assays

Gene expression assays employ cells that have the ability to induce proteins and enzymes that can be measured as an indication of estrogenic activity (Zacharewski 1997). An advantage of this class of assay is that they can activate the genomic, non-genomic and other

pathways not involving the estrogen receptors. Examples of the cells used include Ishikawa and fish hepatocytes that induce alkaline phosphatase (ALP) and vitellogenin respectively (Petit et al., 1997; Matsuoka et al., 2005). A major disadvantage of using these cell lines is that the proteins and enzymes may not be induced in humans and other species. Results from such assays have to be interpreted with caution since they might not actually represent how suspected EDCs might affect the endocrine system of humans. Assays that fall under this category include the primary hepatocyte, BT-474 pS2, and Ishikawa cell- ALP assays. Despite their limitation, gene expression assays have been used to analyze environmental water samples for estrogenic activity (Matsuoka et al., 2005; Rutishauser et al., 2004)

Cell Proliferation Assays

Cell proliferation assays employ human breast cancer cells that are dependent on estrogen and other factors for growth. These assays determine the amount of cell growth after exposing the cell lines to a suspected EDC or sample and compare this value to a positive control. The E-Screen is an example of a cell proliferation assay that can measure estrogenic activity with a limit of detection of 0.27 ng/L (Korner et al., 2001; Folmar et al., 2002). Despite this advantage of sensitivity, this assay is time consuming and expensive; thereby limiting its applications for large-scale screening. Evidence suggests that cell growth can be induced by growth factors, cytokines, and hormones; thereby, leading to false positives (Kinnberg 2003). When compared to the YES, MELN, and T47D-KBluc, analysis of the estrogenic activity in environmental water samples by the E-Screen showed the best correlation to those obtained from chemical analysis of the same samples (Leusch et al., 2010).

1.1.20 Issues Associated with Health Risk Assessment Due to DBPs Estrogenic Activity

In vitro studies have shown different results on the estrogenic activity of DBPs in surface and chlorinated drinking water. One such study analyzed ten chemicals using mammalian cell lines and showed that only 2-chlorophenol was able to activate the genomic pathway of mammalian cells containing the estrogen receptor (Cargouet et al., 2007). The estrogenic activity of 2-chlorophenol occurred at a concentration of 1 mg/L which is several orders of magnitude higher than likely occurrence levels (Sithole & Williams 1986); therefore, this chemical was considered as a weak estrogenic compound. Other DBPs in the Cargouet et al. (2007) study included chloral hydrate, bromoform, and dichloroacetic acid which were shown to not have estrogenic activity consistent with the absence of a phenolic ring on their structures that is thought to be needed to bind to the estrogen receptor.

Another study showed that the estrogenic effect of chlorinated lake water that had been concentrated with an XAD7HP resin had its estrogenic effect increased when compared to the unchlorinated lake water (Itoh et al., 2000). The estrogenic activity of this water was analyzed using mammalian cell lines that had estrogen receptors but without the concentration factor, the estrogenic effect would not have been observed. Another *in vitro* study using mammalian cells showed that the estrogenic effect of WWTP effluent decreased after chlorination. The study also showed no relationship between measured THMs and estrogenic activity of the analyzed samples (Schiliro et al., 2009).

Typical human exposures to DBPs are at very low nanomolar concentrations and so it has been hypothesized that any human health risk is unlikely to be attributed to a single DBP species. Even if it were possible to obtain an endocrine disruptor ranking potential for all the DBPs formed during drinking water disinfection, this chemical-by-chemical approach would

not indicate the effects of interactions between the individual species nor represent the endocrine disruption of the unidentified fraction. Despite this limitation, some studies have been undertaken to determine the estrogenic activity of DBPs when acting alone. *In vitro* studies showed that bromoform, bromodichloromethane, dibromochloromethane, dichloroacetic acid, trichloroacetic acid, dichloroacetonitrile, trichloroacetonitrile, chloral hydrate, and dichloromethane are DBPs that did not induce estrogenic activity (Nishihara et al., 2000; Cargouet et al., 2007). Despite this, it is possible that the complex mixture of negative and weak estrogenic DBPs (such as 2-chlorophenol) found in drinking water might elicit a higher estrogenic and possibly anti-estrogenic response. This assumption is based on results from *in vitro* studies that showed weak estrogenic compounds inducing significant responses when acting in the presence of other compounds (Kortenkamp et al., 2002; Manabe et al., 2006).

1.2 Research Questions

Some chemicals found in surface and groundwater have the potential to alter the endocrine system of fish/aquatic organisms and numerous studies have confirmed this (Jobling & Tyler 2003; Vajda et al., 2008; Renner 2009; Bortone & Davis 1994). The issue of endocrine disruption has been a public health concern over the last 35 years and measures involving protection of drinking water and its sources have been implemented by the U.S. EPA. Drinking water treatment has been shown to remove some EDCs from finished water; however, a different suite of compounds known as DBPs is generated during the process of disinfection (Rook 1974; Krasner et al., 2006). Concern about the health effects associated with these compounds prompted the U.S. EPA to establish MCLs for some of them. A lot of concern still remains about the unregulated DBPs with some research suggesting that they

are more cytotoxic and genotoxic than those which are regulated (Plewa et al., 2002; 2007). At the present time, over 600 DBPs have been identified but they account for less than 50% of total organic halide (TOX) produced during chlorination. Even less is known about those DBPs formed when alternative disinfectants and their combinations are used (Krasner et al., 2006; Richardson et al., 2007).

Humans are continuously exposed to DBPs by various routes, one of which is ingestion of treated drinking water yet very few studies have been conducted to determine their estrogenic activity even as single compounds (Cargouet et al., 2007; Itoh et al., 2000; Nishihara et al., 2000). Despite showing no estrogenic response *in vitro*, dibromoacetic acid is a suspected estrogenic endocrine disruptor as shown from an *in vivo* study (Goldman & Murr 2003). In order to address estrogenic activity of complex mixtures of DBPs found in drinking water, it is necessary to first measure their activity as single compounds. Results from such analysis would show if research efforts need to be placed on certain regulated or unregulated DBPs that might induce human endocrine response.

1.3 Research Objectives

The hypotheses that validate the completion of this research are:

1) The estrogenic activity of chemicals released into drinking water sources can be measured by the YES assay as an initial screening tool.
2) The presence of electronegative halogens in DBPs might induce estrogenic activity.

These hypotheses will be tested by the following experimental objectives

1) Demonstrate the use and applicability of the YES assay to determine the estrogenic activity of DBPs in drinking water.
2) Generate a relative rank order of potency of DBPs using the YES assay.
3) Compare the estrogenic activity of DBPs containing halogens to their non-halogenated counterparts.

2.0. MATERIALS AND METHODS

2.1 Materials

EDC and DBP Standards

Dichloroacetonitrile (95%), dibromoacetic acid (90%), mucochloric acid (99%), and dibromoacetonitrile (95%) were purchased from Aldrich (Milwaukee, WI). 2-bromoacetamide (98%), 2,4-dichlorophenol (99%), 4-nonylphenol (99%) and 2-chlorophenol (98+ %) were purchased from Acros Organics (Fair Lawn, NJ). Estradiol, 17-α-ethynylestradiol (98%), Estriol (99%), iodoacetic acid ($\geq$ 99.5%), 2,4,6-trichlorophenol (98%), and ethanol ($\geq$ 99.5%) were purchased from Sigma Aldrich (St.Louis, MO). Trichloroacetic acid (> 95%), acetonitrile (99.9%), and acetic acid (99.9%) were purchased from Fisher Scientific (Fair Lawn, NJ), while chloral hydrate (95%) was purchased from Ultra Scientific (North Kingstown, RI). Laboratory grade water (LGW) was prepared in-house from a Pure Water Solutions (Hillsborough, NC, USA) system which pre-filters inlet 7 MΩ deionized water to 1 μm, removes residual disinfectants, reduces total organic carbon to less than 0.2 mg/L with an activated carbon resin, and removes ions to 18 MΩ with mixed bed ion-exchange resins. The reagents and other materials used for the YES assay are described in Appendix A

Preparation of stock, primary dilution, and working standard solutions

DBP stock and primary dilution solutions were prepared in ethanol at a target concentration as shown in Table 2.1. The primary dilution solutions were used as a source for

working standard solutions prepared in 10% ethanol at concentrations that ensured solubility in the liquid matrix used as shown in Table 2.2. All stock and primary dilution solutions prepared in ethanol were stored in a freezer (at -15˚C) for a maximum period of 6 months before preparation of new ones, while all working standard solutions in 10% ethanol were freshly prepared on the day of each experiment and stored in a refrigerator at 4˚C. Appendix B shows detailed instructions for working with the YES data generated during this research by using Graphpad Prism 4.03, while the details on the preparation of stock and working standard solutions are shown in Appendix C.

Table 2.1 Concentration of Stock, Primary Dilution and Working Standard Solutions for DBPs

DBP	Stock (mg/L)	1˚Dilution (mg/L)	Working Solution (mg/L)
2,4-dichlorophenol	10300	N/A	278
2,4,6-trichlorophenol	10320	N/A	103
2-bromoacetamide	10110	1011	50
2-chlorophenol	10020	N/A	1002
Chloral hydrate	1000	N/A	10
Dibromoacetic acid	10110	N/A	404
Dibromoacetonitrile	10910	1091	50
Dichloroacetonitrile	10020	1002	50
Iodoacetic acid	10540	1054	50
Mucochloric acid	10060	1006	50
Trichloroacetic acid	13110	N/A	354

Stock: Stock solution prepared in ethanol; 1˚Dilution: Primary dilution standard prepared in ethanol; Working Solution: Working standard solution prepared in 10% ethanol; N/A: Not applicable because working standard solutions were made directly from stock solutions.

Table 2.2 Physical Properties of Target DBPs

DBP	[a]Solubility (g/L)	[b]Solubility (mM)	[c]Vapor Pressure (Torr)
2,4-dichlorophenol	0.47	3	0.136
2,4,6-trichlorophenol	0.32	2	0.0177
2-bromoacetamide	70	507	0.00638
2-chlorophenol	2.4	19	0.875
Chloral hydrate	43	260	25.6
Dibromoacetic acid	1000	4590	0.0144
Dibromoacetonitrile	6.2	31	2.10
Dichloroacetonitrile	13	118	21.7
Iodoacetic acid	1000	5378	0.00329
Mucochloric acid	1000	5919	0.00186
Trichloroacetic acid	1000	6120	0.170

[a]SciFinder For Academics, American Chemical Society, 2011.
[b]Solubility: Solubility at pH 7 in pure water at 25˚C (See Appendix C, section III).
[c]Vapor Pressure: Vapor pressure at 25˚C (See Appendix C, section III).
Note that the pH of the liquid matrix (10% ethanol) was 7.06.

2.2 Methods

2.2.1 The Recombinant Yeast Estrogen Screen

The YES assay was performed according to the method by Routledge and Sumpter (1996) with modifications employed as shown in the standard operating procedure (SOP) in Appendix A (Chen et al., 2007). The yeast cells (*Saccharomyces cerevisiae*) are stably transfected with the human estrogen receptor (hERα) and were kindly provided by Dr. Sumpter at Brunel University in the United Kingdom. Also present in the cells are an estrogen responsive element (ERE) and a plasmid that contains the *Lac-Z* gene that produces the enzyme β-galactosidase. The binding of a receptor-ligand complex to the ERE causes the *Lac-Z* gene to undergo transcription, thereby producing β-galactosidase that is released into the assay medium. This enzyme metabolizes the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (o-NPG) that is normally colorless into a yellow product (o-nitrophenol) that can be measured at an absorbance at 450 nm.

Assay Procedure

Using an 8 multichannel pipettor, 100 μL of 10% ethanol is placed in each of the wells of a deep 96 deep well 1 mL plate except those in the first column. 200 μL of the working standard solution of the selected chemical or sample to be tested is added to two wells in the first column, after which 100 μL from each of the well is pipetted into the adjacent well in the next column by using an 8 multichannel pipettor. The content of the well is mixed gently and 100 μL is then transferred to the adjacent well in the next column and the process of mixing and transfer repeated for each subsequent column, thereby producing a 1:2 serial dilution of the solution in the adjacent well. In order to maintain an equal sample volume in each well, 100 μL is pipetted to waste from each well in the final column. The first two rows of each plate contain E2 as the positive control while the last column is used as a negative control that contains no test chemical or sample but only 100 μL of 10% ethanol. 300 μL of diluted yeast culture is then added to each well after which the plate is sealed with a plate sealing film and incubated at 30˚C for 3 days while shaken at approximately 220 rpm on a shaker table.

After incubation, 50 μL of solution containing Z-buffer and 2-mercaptoethanol (βME) is added to each well and shaken at 300 rpm for approximately 3 to 5 minutes on a shaker table at room temperature. 400 μL of assay buffer containing Z-buffer, βME, and o-NPG is added to each well before incubating the plate at 30˚C for 20 minutes while shaking at approximately 220 rpm. After incubation, 200 μL of 1 M sodium carbonate solution is added to each well to stop the reaction of β-galactosidase with the assay buffer. At this point, the solutions with the ability to induce estrogen receptor (ER) – mediated gene expression will have produced a yellow product (o-nitrophenol). The 96 deep well 1 mL plate is

subsequently centrifuged at 3000 rpm for 10 minutes after which 100 μL of clear supernatant from each well is transferred into a sterile flat 96 well flat bottom microplate. The new plate is then read on a plate reader in order to determine the optical density (OD) at 450 nm ($OD_{450\ nm}$). This value, after subtracting that of the average of the negative control, is then used as a measure of estrogenic activity (indicated as corrected absorbance) and plotted against the molar concentration of the chemical in order to generate a concentration dose or dose-response curve that would provide results on the effective concentration that provides 50% of the maximal observed response (EC_{50}) for the respective chemicals analyzed.

One of the concerns with the YES assay is that of toxicity of the tested chemical towards the yeast cells. Cellular toxicity is assessed by additional processing of the 96 deep well 1 mL plate containing the processed samples or chemicals of interest. The solution in each well of the original plate is resuspended with an 8 multichannel pipettor to allow the yeast cells that had been settled during centrifugation to be mixed with the supernatant. 100 μL of this resuspended solution is then transferred to a sterile 96 well flat bottom microplate and its optical density measured at 600 nm ($OD_{600\ nm}$); the wavelength commonly used to measure turbidity. If the $OD_{600\ nm}$ value for any well containing the tested chemical was at least 10% less than that of the negative control, this is noted as an indication of cytotoxicity for the chemical at the concentration in that well (Nishihara et al., 2004). During data analysis, such cytotoxic concentrations are excluded from the data set used to plot the dose response curves for estrogenic activity for each tested chemical.

Assessment of Estrogenic Activity

Studies have showed that compounds such as phenazone, paracetamol, cimetidine (Fent et al., 2006), dibromoEE2 (Flores & Hill 2008) and *p*-dichlorobenzene (Versonnen et

al., 2003) did not exhibit a full dose response curve when analyzed for estrogenic activity using the YES assay. Such sub-maximal response curves make it impossible to generate an accurate EC_{50} that could subsequently be used to compare relative potencies between chemicals and E2. In order to address this issue, an alternate criterion was used for determining whether a chemical demonstrated estrogenic activity with the YES assay based on that used in a study of 517 chemicals by Nishihara et al. (2000). Positive estrogenic activity was identified when a test chemical solution exhibited estrogenic activity at an absorbance that was at least 10% of the saturated response of 10^{-7} M E2 (i.e. the EC_{10} of E2). This saturated response of E2 represents the flat upper portion of its dose response curve where o-nitrophenol would possibly not be formed as a result of β-galactosidase not being produced by the yeast cells after interaction with chemicals being tested. At this point, estrogen receptors on the yeast cells are fully occupied by the chemicals being assayed. Another reason for a saturated response could be that the o-NPG in the assay medium is completely used up. At this point, produced β-galactosidase would not have any o-NPG to react with so no o-nitrophenol would be formed. This criterion to determine if a chemical was estrogenic was first tested on 2,4-dichlorophenol since the Nishihara et al. (2000) study had demonstrated it as a positive estrogenic compound.

The relative potencies of each tested chemical were also determined and expressed as estradiol equivalents (EEQ). EEQ was calculated by dividing the EC_{50} for E2 by the EC_{50} for each chemical tested in order to compare its relative estrogenicity to E2. The use of the EC_{50} for determination of EEQ was only used when the tested chemical displayed a dose response curve that was parallel to that of E2, while those that displayed sub-maximal response curves were compared using the 10% response level (EC_{10}) (Sumpter et al., 2001).

Quality Assurance

The DBP, E2 working standard and 10% ethanol solution used for each assay were prepared in amber glass vials on the day of each experiment to avoid possible degradation and volatilization. Each chemical was analyzed in duplicate and had a positive E2 control/negative control on each plate. Each DBP was tested at least twice except iodoacetic acid. In order to avoid false positive results due to the reaction of the tested chemical with o-NPG to form o-nitrophenol, the assay was performed (as specified under the assay procedure) by using the highest concentration of the chemical without the addition of the diluted yeast solution. The formation of a yellow product after completion of the assay in the absence of yeast cells would be taken as a false positive response for the estrogenic activity of the tested chemical and this would rule out the use of the YES assay for the selected tested chemicals showing such a response.

3.0 RESULTS

3.3.1 Estrogenic Activity of Known EDCs towards the YES Assay

Use and applicability of the YES assay

The sensitivity and reproducibility of the YES assay was determined by measuring responses to increasing concentrations of E2 ($2.40x10^{-11}$ - $1.25x10^{-8}$ M), EE2 ($6.10x10^{-12}$ - $1.25x10^{-8}$ M), E3 ($1.10x10^{-9}$ - $2.25x10^{-6}$ M) and 4-nonylphenol ($2.47x10^{-7}$ - $6.33x10^{-5}$ M). These concentration ranges were chosen based on those reported in the Chen et al. (2007) study. The sigmoid-shaped concentration response curves (otherwise referred to as dose-response curves) were fitted to a symmetric logistic function using Graphpad Prism 4 software (Version 4.03, La Jolla, CA) and plotted. As shown in Figures 3.1 to 3.4, all these chemicals induced estrogenic activity in a dose dependent manner.

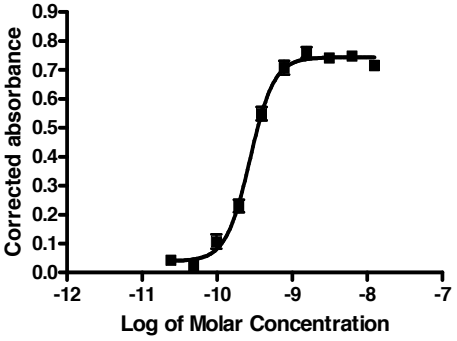


Figure 3.1 Dose Response Curve for Estradiol (E2) in the YES Assay

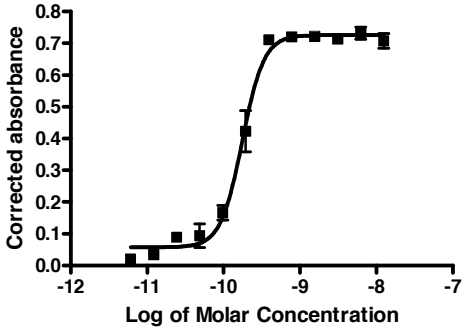


Figure 3.2 Dose Response Curve for 17-α-ethinylestradiol (EE2) in the YES Assay

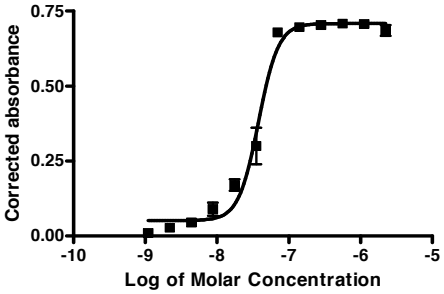


Figure 3.3 Dose Response Curve for Estriol (E3) in the YES Assay

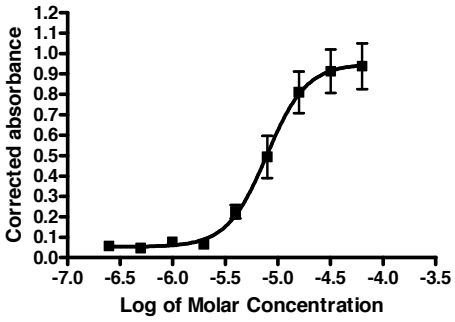


Figure 3.4 Dose Response Curve for 4-nonyphenol (4-NP) in the YES Assay

The effective concentration generating a response halfway between the baseline and maximal response is designated as the EC_{50} and the values obtained during these experiments were comparable to those in the literature as shown in Table 3.1.

Table 3.1 EC_{50} Values for Steroid Hormones and 4-NP in this Study Compared to Previous Studies

Chemical	Current EC_{50} (M)	[a]Lower 95th % CI (M)	[a]Upper 95th % CI (M)	Literature EC_{50} (M)	Reference
E2	2.75×10^{-10}	2.55×10^{-10}	2.97×10^{-10}	1.80×10^{-10}	Beck et al., 2006
				2.10×10^{-10}	Rutishauser et al., 2004
				1.70×10^{-10}	Flores & Hill 2008
EE2	1.76×10^{-10}	1.61×10^{-10}	1.93×10^{-10}	2.40×10^{-10}	Beck et al., 2006
				1.80×10^{-10}	Rutishauser et al., 2004
				1.60×10^{-10}	Flores & Hill 2008
E3	3.89×10^{-8}	3.50×10^{-8}	4.33×10^{-8}	2.20×10^{-8}	Beck et al., 2006
				8.90×10^{-8}	Rutishauser et al., 2004
[b]E2	1.38×10^{-10}	1.25×10^{-10}	1.53×10^{-10}	Same for E2 above	
[b]4-NP	7.84×10^{-6}	6.58×10^{-6}	9.34×10^{-6}	7.00×10^{-6}	Beck et al., 2006
				8.40×10^{-6}	Rutishauser et al., 2004

[a]Lower/Upper 95th % CI: See Appendix B on generation of Lower and Upper 95th % confidence interval by Graphpad Prism 4 software.
[b]4-NP was analyzed separately from the steroids shown in Table 3.1, so its concurrent positive control ([b]E2) EC_{50} value is different.

The relative potency also known as estradiol equivalents (EEQ) is the total concentration of estrogenic active compounds in an environmental sample that is normalized to E2 and is calculated by dividing the EC_{50} of E2 by that of a sample (Schiliro et al., 2009). Table 3.2 shows the EEQ for the compounds analyzed (relative to E2) and a comparison to those values in the literature. When compared to the estrogenic activity of E2, the most active chemical is the synthetic hormone EE2 followed by E3, while 4-NP showed the lowest activity.

Table 3.2 EEQ Values for Steroid Hormones and 4-NP in this Study Compared to Previous Studies

Chemical	Current EC_{50} (M)	Current EEQ	Literature EEQ	Reference
E2	2.75×10^{-10}	1.00	[a]N/A	
EE2	1.76×10^{-10}	1.56	1.25	Beck et al., 2006
			1.19	Rutishauser et al., 2004
			1.06	Flores & Hill 2008
E3	3.89×10^{-8}	7.07×10^{-3}	5.93×10^{-3}	Beck et al., 2006
			2.40×10^{-3}	Rutishauser et al., 2004
E2	1.38×10^{-10}	1.00	[a]N/A	
4-NP	7.84×10^{-6}	1.76×10^{-5}	1.85×10^{-5}	Beck et al., 2006
			2.50×10^{-5}	Rutishauser et al., 2004

[a]N/A: Not applicable.

Validation of Nishihara et al. (2000) Criterion for Determination of Estrogenic Activity

The EC_{50} values were used in determination of the EEQ for the respective EDCs in Table 3.2 since those chemicals displayed a full dose response curve. 2,4-dichlorophenol displayed almost a full dose response curve across a concentration range from 4.16×10^{-7} to 4.26×10^{-4} M as shown in Figure 3.5. Based on the Nishihara et al. (2000) study criterion, this chemical is estrogenic since its maximal corrected absorbance of approximately 0.5 (as shown in Figure 3.5) is greater than 10% of the corrected absorbance of 10^{-8} M E2 (approximately 0.1 as shown in Figure 3.6). Results from the Nishihara et al. (2000) study also showed that positive estrogenic compounds all had reported EC_{10} values greater than the EC_{10} for 10^{-7} M E2. The EC_{10} for 2,4-dichlorophenol in this study was calculated to be at a concentration of 9.21×10^{-5} M, which is higher than that of E2 assayed on the same 96 deep well 1 mL plate (5.40×10^{-11} M). These results validate the Nishihara et al. (2000) study criterion which showed an EC_{10} for 2,4-dichlorophenol as 4×10^{-5} M and for E2 as 3×10^{-10} M. It should be noted that the EC_{10} reported in this current study is lower than that in

the Nishihara et al. (2000) study since a lower E2 concentration of 10^{-8} M was used compared to 10^{-7} M used in the Nishihara et al. (2000) study.

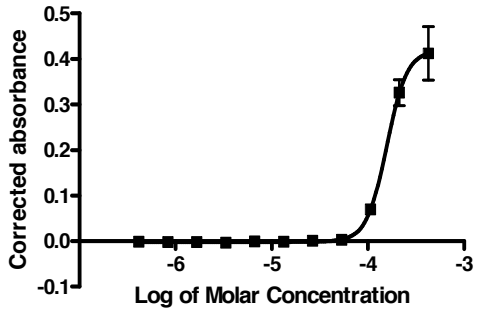


Figure 3.5 Dose Response Curve for 2,4-dichlorophenol (2,4-DCP) in the YES Assay

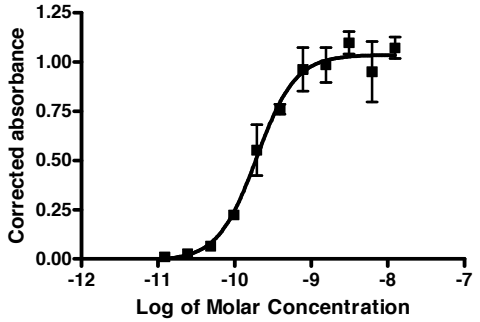


Figure 3.6 Dose Response Curve for Estradiol (E2) Analyzed Simultaneously on the same plate with 2,4-dichlorophenol in the YES Assay

Based on numerous positive E2 controls assayed during each experiment, the observed saturated response for E2 was always between 0.8 and 1.0 absorbance units as shown in Figures 3.7 to 3.10. A tested chemical was considered as estrogenic if its saturated response was at least 0.08 absorbance units or greater.

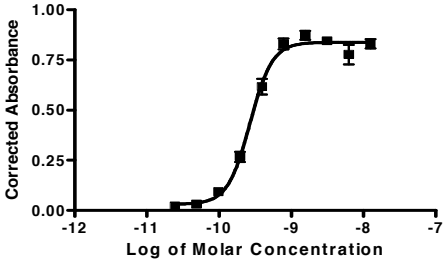


Figure 3.7 E2: Saturated Response (0.853)

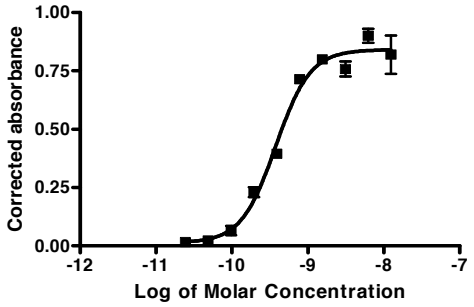


Figure 3.8 E2: Saturated Response (0.903)

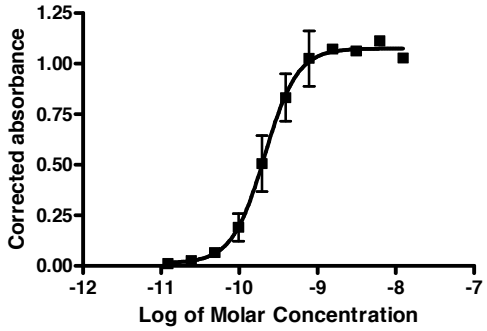


Figure 3.9 E2: Saturated Response (1.044)

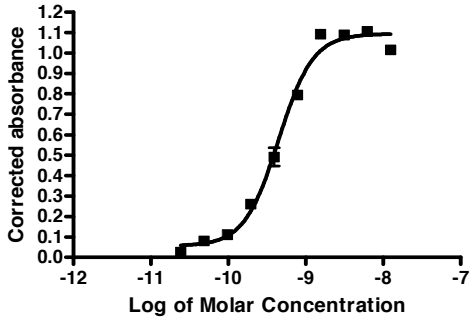


Figure 3.10 E2: Saturated Response (1.018)

Quality Assurance

Table 3.3 shows the EC_{50} values for the positive control (E2) calculated from 7 independent experiments. The mean EC_{50} of these 7 experiments, standard deviation, and relative standard deviation (RSD) value is 2.47 x 10^{-10} M, 2.17 x 10^{-11} M, and 8.76 percent, respectively. These EC_{50} values (shown in Table 3.3) in addition to the mean EC_{50} value

generated during this study were in the same order of magnitude as those reported in the literature as shown in Table 3.1.

Table 3.3 Estradiol (E2) EC_{50} Values for 7 Independent Experiments

Experiment #	EC_{50} (M)	[a]Lower 95th % CI (M)	[a]Upper 95th % CI (M)
1	2.54×10^{-10}	1.78×10^{-10}	3.63×10^{-10}
2	2.11×10^{-10}	1.81×10^{-10}	2.46×10^{-10}
3	2.50×10^{-10}	1.97×10^{-10}	3.16×10^{-10}
4	2.19×10^{-10}	2.01×10^{-10}	2.38×10^{-10}
5	2.67×10^{-10}	2.45×10^{-10}	2.92×10^{-10}
6	2.72×10^{-10}	2.51×10^{-10}	2.94×10^{-10}
7	2.58×10^{-10}	2.40×10^{-10}	2.77×10^{-10}

[a]Lower/Upper 95th % CI: See Appendix B on generation of Lower and Upper 95th % confidence interval by Graphpad Prism 4 software.

3.3.2 Estrogenic Activity of Selected DBPs towards the YES Assay

Objective 1 and 2: Determine the estrogenic activity of DBPs by using the YES assay and generate a relative rank order of potency

The DBPs used in this study were selected based on their occurrence in drinking water and relevance based on toxicological health effects as reported from *in vivo*, *in vitro* and epidemiological studies. Figures 3.11 to 3.20 shows the dose-response curves for the selected DBPs analyzed during this research.

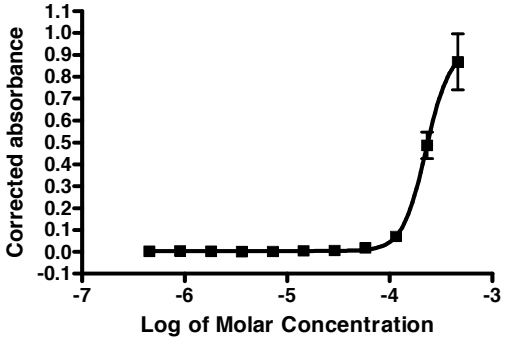


Figure 3.11 Dose Response Curve for Dibromoacetic acid in the YES Assay

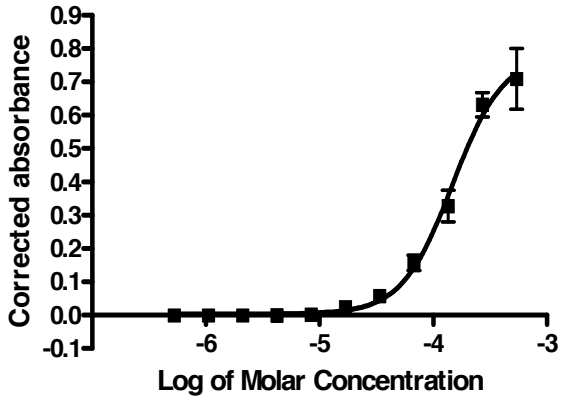


Figure 3.12 Dose Response Curve for Trichloroacetic acid in the YES Assay

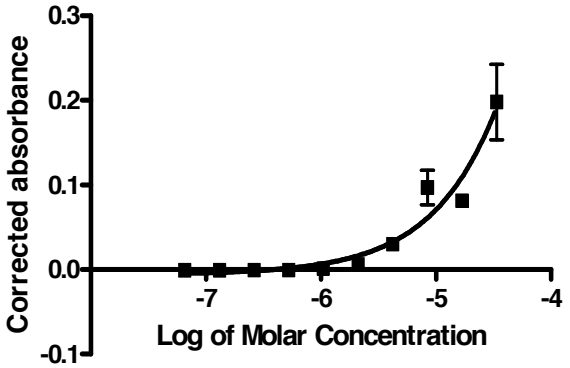


Figure 3.13 Dose Response Curve for Iodoacetic acid in the YES Assay

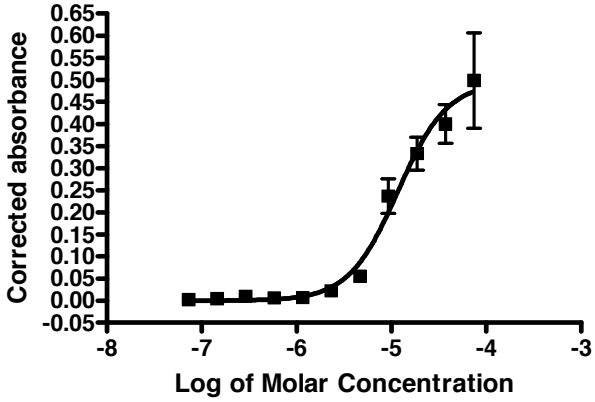


Figure 3.14 Dose Response Curve for Mucochloric acid in the YES Assay

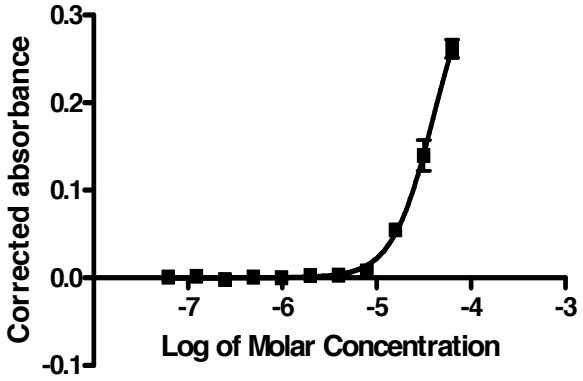


Figure 3.15 Dose Response Curve for Dibromoacetonitrile in the YES Assay

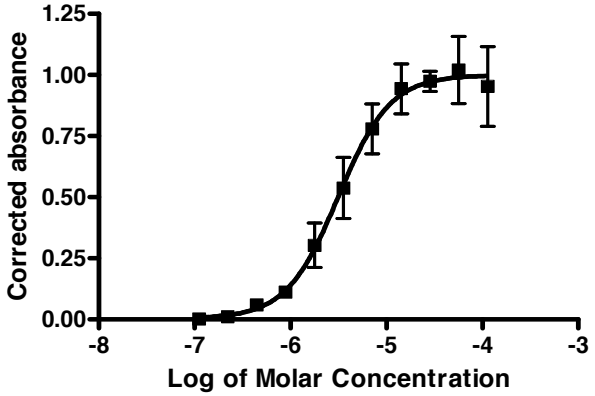


Figure 3.16 Dose Response Curve for Dichloroacetonitrile in the YES Assay

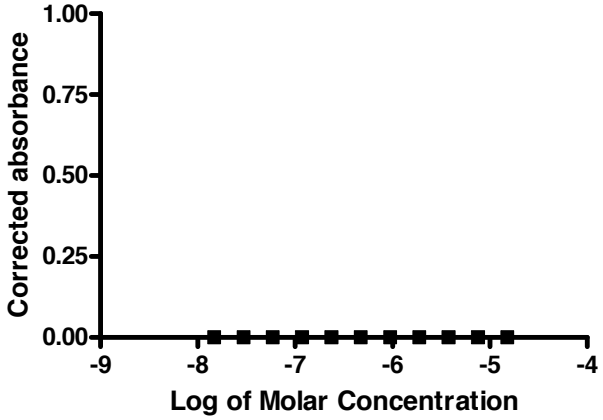


Figure 3.17 Dose Response Curve for Chloral hydrate in the YES Assay

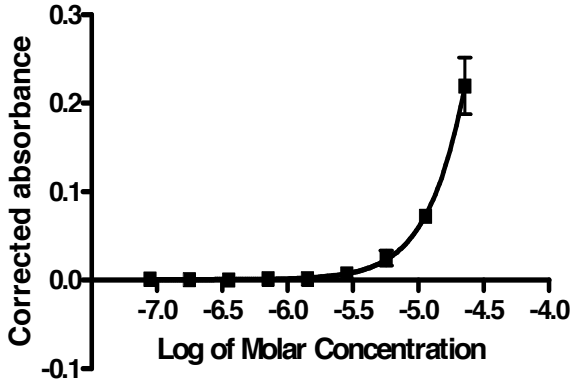


Figure 3.18 Dose Response Curve for 2-bromoacetamide in the YES Assay

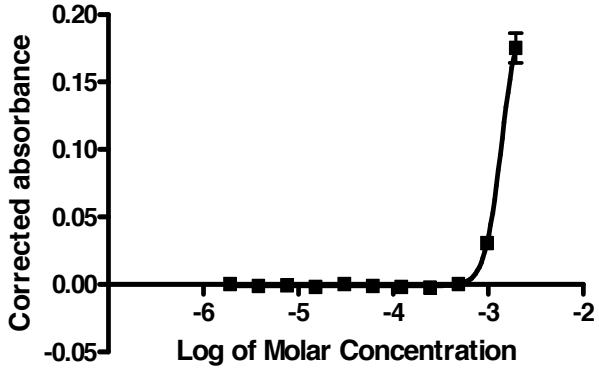


Figure 3.19 Dose Response Curve for 2-chlorophenol in the YES Assay

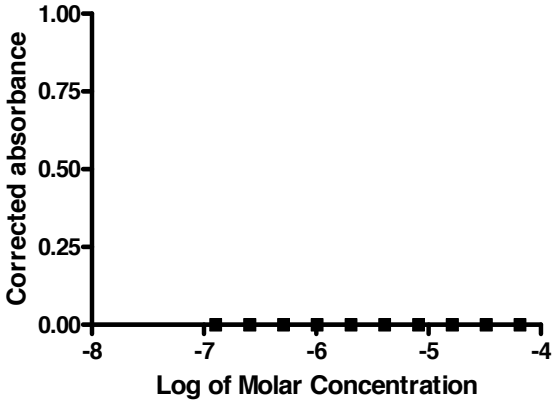


Figure 3.20 Dose Response Curve for 2,4,6-trichlorophenol in the YES Assay

The concentrations at the higher end ranges of DBPs assessed during this screening were not cytotoxic to the yeast cells except those for 2-bromoacetamide, iodoacetic acid and

2,4,6-trichlorophenol. The yeast cell densities (measured at $OD_{600\ nm}$) were recorded for the wells containing the negative controls and each DBP dilution. Any significant reduction in this absorbance was taken as an indication of the yeast cell growth inhibition or cytotoxicity. Table 3.4 summarizes these values and shows cytotoxicity at the highest concentrations of 2-bromoacetamide, iodoacetic acid and 2,4,6-trichlorophenol. Previous studies with the YES assay have used this measurement as an indication of acute toxicity and mortality of the yeast cells (Beck et al., 2006; Versonnen et al., 2003; Hamblen et al., 2003; Schultz et al., 2000).

Table 3.4 Summary of Measurements for DBPs with the YES assay

Compound	[a]Highest Conc Conc. (M)	[b]Average $OD_{600\ nm}$	[c]Neg Control Average $OD_{600\ nm}$	Cytotoxicity (%)
2,4-dichlorophenol	4.26×10^{-4}	0.282	0.247	N/A
2,4,6-trichlorophenol	6.53×10^{-5}	0.060	0.213	72
2-Bromoacetamide	9.07×10^{-5}	[d]0.101	0.284	64
	4.53×10^{-5}	[e]0.130	0.284	54
2-chlorophenol	1.95×10^{-3}	0.237	0.247	N/A
Chloral hydrate	1.51×10^{-5}	0.200	0.191	N/A
Dibromoacetic acid	4.64×10^{-4}	0.266	0.247	N/A
Dibromoacetonitrile	6.31×10^{-5}	0.210	0.186	N/A
Dichloroacetonitrile	1.14×10^{-4}	0.248	0.214	N/A
Iodoacetic acid	6.73×10^{-5}	0.095	0.284	67
Mucochloric acid	7.44×10^{-5}	0.280	0.284	N/A
[f]Trichloroacetic acid	5.41×10^{-4}	0.242	0.295	N/A

N/A: No significant cytotoxic activity observed.
[a]Highest Conc: Highest molar concentration tested.
[b]Average $OD_{600\ nm}$: Average $OD_{600\ nm}$ for DBP, n = 2 wells.
[c]Neg Control Average $OD_{600\ nm}$: Average $OD_{600\ nm}$ for negative control, n = 8 wells for all chemicals except dibromoacetonitrile (n = 7 wells), dichloroacetonitrile (n = 4 wells), and Trichloroacetic acid (n = 4 wells).
[d,e]2 values are shown for 2-bromoacetamide because cytotoxicity was observed at the highest 2 of the 11 concentrations as shown in Figure 3.21.
[f]Trichloroacetic induced 18% yeast cell growth inhibition, this activity did not cause a sudden drop in absorbance as shown in Figure 3.12 ; therefore, 5.41×10^{-4} M was not excluded from the data set used in plotting the dose response curve.

Figures 3.21 and 3.22 show the cytotoxicity dose-response curves for iodoacetic acid and 2-bromoacetamide across a concentration range from 6.57×10^{-8} M to 6.73×10^{-5} M and

8.85 x 10^{-8} M to 9.07 x 10^{-5} M, respectively. Cytotoxic activity of these DBPs to the yeast cells caused the induction of estrogenic activity to stop and this is very obvious by the sudden drop in their corrected absorbance at a particular concentration (6.73 x 10^{-5} M for iodoacetic acid and 4.53 x 10^{-5} to 9.07 x 10^{-5} M for 2-bromoacetamide).

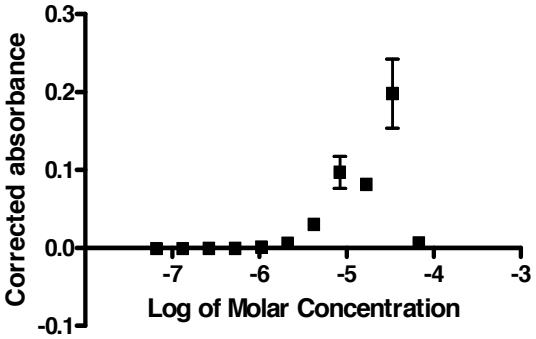


Figure 3.21 Cytotoxic Dose Response Curve for Iodoacetic acid in the YES Assay (absorbance measured at 600nm)

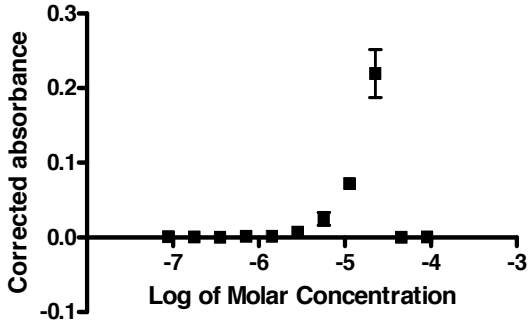


Figure 3.22 Cytotoxic Dose Response Curve for 2-bromoacetamide in the YES Assay (absorbance measured at 600nm)

The cytotoxic activity shown in Figures 3.21 and 3.22 complements the results from previous studies that showed iodoacetic acid inducing cytotoxicity in *Salmonella typhimurium* TA100 and Chinese Hamster Ovary AS52 cells (Plewa et al., 2004), while 2-bromoacetamide induced the same activity in the mammalian cell (Plewa et al., 2008).

Table 3.5 compares the cytotoxicity results for iodoacetic acid and 2-bromoacetamide in this current study using yeast cells with those from the Plewa et al. (2004) study using mammalian cells and the Kargalioglu et al. (2002) study using bacteria.

Table 3.5 Iodoacetic acid and 2-bromoacetamide Cytotoxicity in Bacteria, Yeast, and Mammalian Cells

DBP	Conc. Range Tested (M)	Cytotoxic Conc. (M)	Inhibition (%)
Iodoacetic acid (Yeast)	6.57×10^{-8} - 6.73×10^{-5}	6.73×10^{-5}	67
Iodoacetic acid ([a]Bacteria)	1.00×10^{-4} - 1.00×10^{-3}	3.03×10^{-4}	50
Iodoacetic acid ([b]CHO)	5.00×10^{-7} - 1.20×10^{-5}	2.95×10^{-6}	50
2-Bromoacetamide (Yeast)	8.85×10^{-8} - 9.07×10^{-5}	$\geq 4.53 \times 10^{-5}$	≥ 54
2-Bromoacetamide ([b]CHO)	$1.00 \times 10^{-7} \approx 1.00 \times 10^{-5}$	1.89×10^{-6}	50

[a]Bacteria: *Salmonella typhimurium* TA100; [b]CHO: Chinese Hamster Ovary AS52 cells.

Inhibition in the Plewa et al. (2004) study was expressed as $\%C^{1/2}$ and this represented the concentration of the DBP that reduced the cell density by 50% when compared to that of the concurrent negative control. Table 3.5 also gives the concentration range in which CHO cells in the Plewa et al. (2008) study exposed to 2-bromoacetamide experienced cytotoxicity.

Summary and implication of results for cytotoxic activity of selected DBPs to the yeast cells in this study

2-bromoacetamide and iodoacetic acid caused significant cytotoxic activity to the yeast cells used during this study and the same activity has been reported by *in vitro* studies using mammalian (Chinese Hamster Ovary) cells (Plewa et al., 2004; and 2008). Determination of cytotoxic activity using the CHO cells involves measurement of the CHO cell density that was exposed to a DBP after 72 hours of growth. The DBP concentration that causes a 50% reduction of the CHO cell density when compared to its concurrent negative control is referred to as $\%C^{1/2}$ and this value is used to generate a cytotoxicity relative rank order potency for the analyzed DBPs (Plewa et al., 2004, 2008). The cytotoxic end point

measured with CHO cells is the same as that in the yeast cells used during this study because a reduction of yeast cell density is being measured in both methods. During this study, an optical density reading at 600 nm was taken for a flat 96 well plate containing resuspended yeast cells for each analyzed DBP. A significant reduction in the yeast cell density when compared to the OD 600 readings of the negative control was taken as an indication of cytotoxicity.

Based on cytotoxic activity documented in this current study when analyzed in the concentration range (as shown in Table 3.6), the yeast cells have much more tolerance than CHO cells (and possibly other mammalian cells) for 2-bromoacetamide and iodoacetic acid (see Table 3.7) and as such, cannot be used to accurately predict the quantitative absolute cytotoxic risks in humans. This hypothesis is supported by a study that evaluated the estrogenic activity of selected phytoestrogens using a recombinant yeast strain and MCF7 mammalian cells (Breinholt & Larsen 1998). Genistein (one of the phytoestrogens) in the concentration range of 1.00×10^{-7} - 1.00×10^{-4} M showed no evidence of cytoxicity with yeast cells but with MCF7 mammalian cells, cytotoxicity was observed at concentrations above 1.00×10^{-6} M.

Table 3.6 Range of Concentrations Tested for Selected DBPs Compared to Previous Studies

DBP	Range of Tested Conc. (M) [#,*,@]	Range of Tested Conc. in this Study (M)
Dibromoacetonitrile	[#] 1.00×10^{-6} - 1.00×10^{-5}	6.16×10^{-8} - 6.31×10^{-5}
Dichloroacetonitrile	[#] 2.00×10^{-6} - 1.00×10^{-4}	1.11×10^{-7} - 3.88×10^{-4}
2-Bromoacetamide	[*] $1.00 \times 10^{-7} \approx 1.00 \times 10^{-5}$	8.85×10^{-8} - 9.07×10^{-5}
Dibromoacetic acid	[@] 1.00×10^{-6} - 1.25×10^{-3}	4.53×10^{-7} - 4.64×10^{-4}
Trichloroacetic acid	[@] 1.00×10^{-3} - 4.30×10^{-2}	5.29×10^{-7} - 2.71×10^{-4}
Iodoacetic acid	[#] 5.00×10^{-7} - 1.20×10^{-5}	6.57×10^{-8} - 6.73×10^{-5}

[#]Plewa et al., 2007; *Plewa et al., 2008; [@]Plewa et al., 2002.

Other DBPs such as dibromoacetonitrile, dibromo- and trichloroacetic acid analyzed during this study showed no evidence of the significant cytotoxic activity that was observed when assayed using the CHO cells as shown in Table 3.7. These observations point to a major difference in sensitivity between assays using yeast and mammalian cells, at least for cytotoxicity, and beg caution in the interpretation of estrogenic effects measured by the YES assay towards human health.

Table 3.7 Cytotoxic Concentrations for DBPs Compared to Previous Studies

DBP	Cytotoxic Conc. (M)[a]	Rank Order	Lowest Cytotoxic Conc. in this Study (M)	Rank Order in this Study
Dibromoacetonitrile	[#]2.85×10^{-6}	2	N/A	N/A
Dichloroacetonitrile	[#]5.73×10^{-5}	3	N/A	N/A
2-Bromoacetamide	[*]1.89×10^{-6}	1	[b]4.53×10^{-5}	1
Dibromoacetic acid	[@]5.00×10^{-4}	4	N/A	N/A
Trichloroacetic acid	[@]1.75×10^{-2}	6	N/A	N/A
Iodoacetic acid	[$]$2.95 \times 10^{-3}$	5	[c]6.73×10^{-5}	2

N/A: Not applicable because DBPs were not cytotoxic to the yeast cells.
[a]The values presented for these DBP represent the cytotoxic concentration that causes reduction of the CHO cell density by 50% when compared to the concurrent negative control. [b]2-bromoacetamide caused greater than 54% decrease at 4.53×10^{-5} M. [c]Iodoacetic acid caused a 67% decrease in the yeast cell density at 6.73×10^{-5} M.
[#]Plewa et al., 2007; *Plewa et al., 2008; [@]Plewa et al., 2002; [$]Plewa et al., 2004.

Addressing Possibility of False Positive Results

Possible false positive results from conversion of o-NPG to o-nitrophenol were addressed by incubating the highest concentrations of the DBPs showing no cytotoxic activity in the assay medium without the yeast. The phenolic compounds were not tested since they have the structures needed to bind to the estrogenic receptor and previous studies have confirmed that they induced estrogenic activity (Nishihara et al., 2000; Cargouet et al., 2007). The corrected absorbance for these DBPs are expressed as the average $OD_{450\ nm}$ reading after that of the negative control (0.037 absorbance units) was subtracted. Based on the results shown in Table 3.8, none of the DBPs induced estrogenic activity in the absence

of yeast solution since their corrected absorbance ($OD_{450\ nm}$) did not have a value of at least 0.08 absorbance units.

Table 3.8 Corrected Absorbance of DBPs Assayed without Diluted Yeast Solution

DBP		Concentration (M)	Corrected $OD_{450\ nm}$
Dibromoacetic acid	(n = 8 wells)	4.64×10^{-4}	0.001
Trichloroacetic acid	(n = 8 wells)	5.41×10^{-4}	0.001
Dibromoacetonitrile	(n = 8 wells)	6.31×10^{-5}	0.001
Dichloroacetonitrile	(n = 8 wells)	1.14×10^{-4}	0.001
Mucochloric acid	(n = 8 wells)	7.44×10^{-5}	0

Table 3.9 shows the EC_{50} values for the tested DBPs calculated by the Graphpad Prism software in molar concentrations (M) and these were converted into μg/L concentrations so that a context is provided for occurrence levels in drinking water which are typically in the range of 1 – 10 μg/L (Weinberg et al., 2002; Krasner et al., 2006; McGuire et al., 2002). The table also shows the log of the relative potency of the tested DBPs calculated using their EC_{10} values and that of their concurrent positive control, since not all of them displayed a full dose response curve.

Table 3.9 Comparison of EC_{10}, EC_{50}, and Relative Potencies of DBPs Analyzed

DBP	EC_{50} (M)	EC_{10} (M)	EC_{10} (μg/L)	EC_{10} Relative Potency Log	Rank Order
Estradiol	1.94×10^{-10}	5.40×10^{-11}	1.47×10^{-2}	0	
2, 4-dichlorophenol	1.57×10^{-4}	9.21×10^{-5}	1.50×10^{4}	- 6.23	7
2-chlorophenol	1.38×10^{-3}	8.84×10^{-4}	1.14×10^{5}	- 7.21	9
[a]Dibromoacetic acid	2.26×10^{-4}	1.26×10^{-4}	2.75×10^{4}	- 6.36	8
Estradiol	4.89×10^{-10}	8.89×10^{-11}	2.42×10^{-2}	0	
Mucochloric acid	1.17×10^{-5}	3.16×10^{-6}	5.34×10^{2}	- 4.55	2
2-Bromoacetamide	6.71×10^{-5}	1.89×10^{-5}	2.61×10^{3}	- 5.33	3
Iodoacetic acid	1.73×10^{-3}	1.12×10^{-4}	2.08×10^{4}	- 6.10	6
Estradiol	2.50×10^{-10}	6.15×10^{-11}	1.68×10^{-2}	0	
[a]Trichloroacetic acid	1.48×10^{-4}	4.81×10^{-5}	7.86×10^{3}	- 5.89	5
Dichloroacetonitrile	3.15×10^{-6}	8.02×10^{-7}	8.82×10^{1}	- 4.12	1
Estradiol	1.22×10^{-10}	4.77×10^{-11}	1.30×10^{-2}	0	
Dibromoacetonitrile	4.01×10^{-5}	1.32×10^{-5}	2.62×10^{3}	- 5.44	4

[a]Dibromo- and trichloroacetic acid: DBPs currently regulated by the U.S. EPA.

The results shown in Table 3.9 shows each DBP with its concurrent E2 assayed simultaneously on the same 96 deep well 1 mL plate.

3.3.3 Possible Role of Chlorine and Bromine Atoms in Estrogenic Activity of DBPs

Objective 3: Comparison of the estrogenic activity of DBPs containing chlorine and bromine atoms to their non-halogenated counterparts

The DBPs analyzed during this study showed estrogenic activity despite the absence of a phenolic moiety (except for the chlorophenols). None of the DBPs generated false positive responses by reacting with the assay medium and o-NPG, so their estrogenic activity was estrogen-receptor mediated. In order to determine if bromine and chlorine atoms on selected DBPs played any role in inducing an estrogenic response, acetonitrile and acetic acid were initially assayed at the same molar concentration range used for the haloacetonitriles (dibromoacetonitrile/dichloroacetonitrile) and the haloacetic acids (dibromoacetic/trichloroacetic acid), respectively. Subsequent analysis was carried out in a much higher molar concentration range to determine if acetonitrile and acetic acid induced any estrogenic activity in the YES assay.

Figures 3.23 and 3.24 shows the dose response curves generated for acetonitrile and dichloroacetonitrile assayed in the concentration range $3.70x10^{-7}$ - $3.07x10^{-4}$ M and $1.11x10^{-7}$ - $1.14x10^{-4}$ M, respectively. Figures 3.25 and 3.26 shows the dose response curves for acetic acid and dibromoacetic acid analyzed in the concentration range of $1.64x10^{-6}$ - $1.68\ x10^{-3}$ M and $4.53x10^{-7}$ - $4.64x10^{-4}$ M, respectively. Figures 3.27 and 3.28 show acetonitrile ($4.62\ x\ 10^{-5}$ - $4.72\ x\ 10^{-2}$ M) and acetic acid ($4.26\ x\ 10^{-5}$ - $4.34\ x\ 10^{-2}$ M) assayed at a much higher molar concentration.

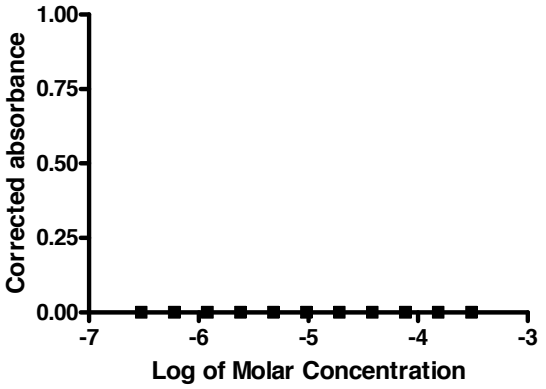


Figure 3.23 Dose Response Curve (lower concentration range) for Acetonitrile in the YES Assay

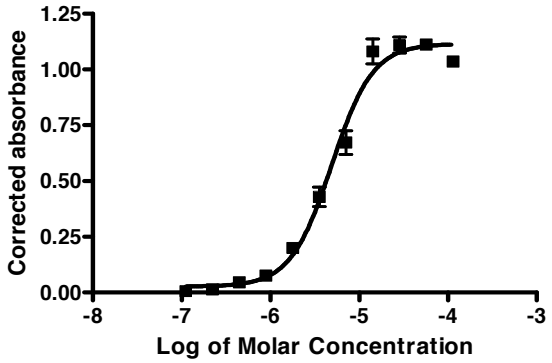


Figure 3.24 Dose Response Curve (lower concentration range) for Dichloroacetonitrile in the YES Assay

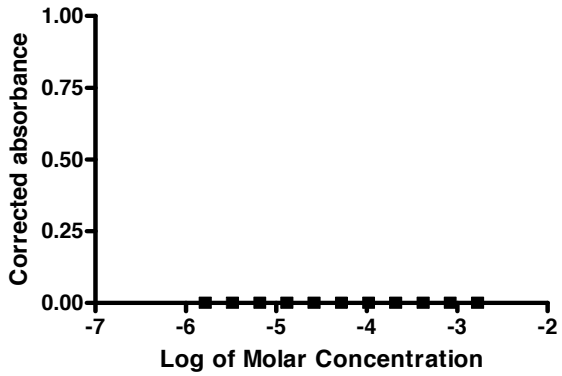


Figure 3.25 Dose Response Curve (lower concentration range) for Acetic acid in the YES Assay

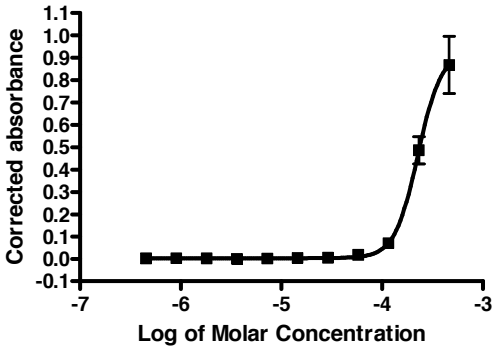


Figure 3.26 Dose Response Curve (lower concentration range) for Dibromoacetic acid in the YES Assay

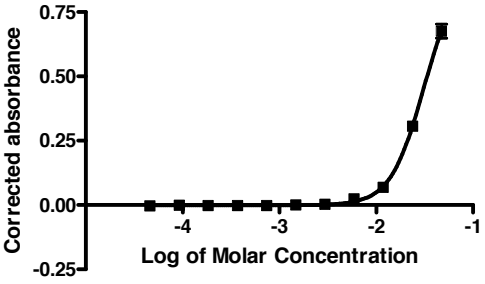


Figure 3.27 Dose Response Curve (higher concentration range) for Acetonitrile in the YES Assay

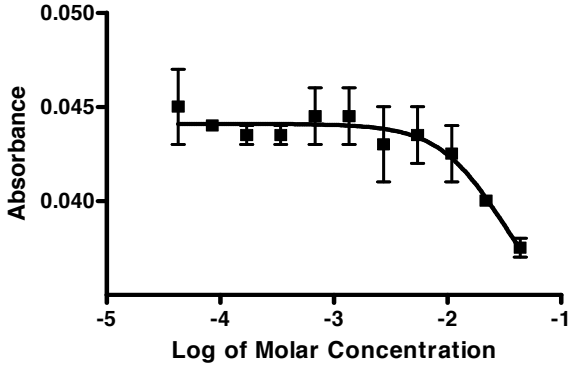


Figure 3.28 Dose Response Curve (higher concentration range) for Acetic acid in the YES Assay

Figure 3.28 shows a decline in absorbance at 450 nm at the higher concentrations of acetic acid and this was due to cytotoxicity of the yeast cells beginning at 1.09×10^{-2} M. Measurement of the optical density at 600 nm ($OD_{600\ nm}$) confirmed this and the various

molar concentrations of this chemical that reduced the yeast cell density when compared to the negative control are presented in Table 3.10.

Table 3.10 Cytotoxic Activity of Acetic Acid to Yeast Cells

[a]Neg. Control $OD_{600\ nm}$	[b]Acetic acid Average $OD_{600\ nm}$	Cytotoxic Conc. (M)	[c]Inhibition (%)
0.215	0.058	4.34×10^{-2}	73
	0.160	2.18×10^{-2}	26
	0.183	1.09×10^{-2}	15

[a]Neg. Control $OD_{600\ nm}$: Average of negative control, n = 8 wells.
[b]Acetic acid Average $OD_{600\ nm}$, n = 2 wells.
[c]Inhibition (%) = (Average $OD_{600\ nm}$ for negative control – Average $OD_{600\ nm}$ for chemical tested)/ (Average $OD_{600\ nm}$ for negative control) multiplied by 100.

Summary of results for estrogenic activity of DBPs containing halogens to their non-halogenated counterparts

The results presented in this study determined the relative potency of each DBP and phenolic compounds by using their EC_{10} values since most of them did not display a full dose response curve. Most of the analyzed DBPs begin to show estrogenic activity only at concentrations much higher than are typically found in drinking water. Relative rank order potency was also generated by comparing their EC_{10} values to that of the concurrent positive control, E2. Using this approach, dichloroacetonitrile (with an EC_{10} of 8.02×10^{-7} M) was the most estrogenic DBP among those tested while dibromoacetonitrile (with an EC_{10} of 1.32×10^{-5} M) was ranked fourth. In order to better determine the role the halogen plays in determining estrogenicity, the non-halogenated backbone of the molecule (acetonitrile) was assessed for estrogenicity in the same concentration range (3.70×10^{-7} - 3.07×10^{-4} M) as the halogenated compounds. No response was observed and the chemical was also not cytotoxic in this range. In a second round of tests in a higher concentration range of 4.62×10^{-5} - 4.72×10^{-2} M, estrogenic activity was observed (with an EC_{10} of 1.29×10^{-2} M) but it is clear that the bromine and chlorine atoms were responsible for the estrogen-mediated

response observed in dichloro- and dibromoacetonitrile and that chlorine indeed initiates a higher estrogen response than bromine.

A similar observation for the analyzed haloacetonitriles was made for the regulated dibromoacetic acid (ranked 8th in the YES evaluation with an EC_{10} of 1.26 x 10^{-4} M) and trichloroacetic acid (ranked 5th with an EC_{10} of 4.81 x 10^{-5} M). The unregulated iodoacetic acid ranked 6th in this study (with an EC_{10} of 1.12 x 10^{-4} M) was also selected for analysis since it has been reported as the most potent genotoxic DBP (Plewa et al., 2004) and no study has, so far, shown results for its estrogenic activity. Further analysis was conducted to determine if the halogen on these haloacetic acids was responsible for their estrogenic activity. The non-halogenated molecule (acetic acid) was analyzed in the same concentration range (1.64 x 10^{-6} - 1.68 x 10^{-3} M) of iodo-, dibromo- and trichloroacetic acid that showed estrogenic activity but no activity nor cytotoxicity was observed. Acetic acid was further analyzed in the concentration range of 4.26 x 10^{-5} - 4.34 x 10^{-2} M in order to determine if it was estrogenic but no activity was observed; however, cytotoxicity was observed in the concentration range of 1.09 x 10^{-2} - 4.34 x 10^{-2} M. The negative estrogenic response by acetic acid results rules out the possibility that it contributed to the estrogenic activity shown by the haloacetic acids analyzed during this study; therefore, the presence of chlorine and bromine appears to activate the genomic pathway in the yeast cells.

4.0 DISCUSSION

4.1 Estrogenic Activity of Selected DBPs and Phenolic Compounds towards the Yeast Estrogen Screen

The YES assay was chosen for the initial screening of DBP estrogenic activity because it is a relatively inexpensive and high-throughput tool compared to other *in vitro* assays such as the E-Screen and ER-CALUX. It also has some value in evaluating comparative effects of target chemicals on mammalian cells since the human estrogen estrogen receptor (hERα) is integrated into the yeast cells. Furthermore, since endogenous steroid hormone receptors are absent in the yeast cells, a positive estrogenic response with this assay is taken as estrogen-receptor mediated. Prior to the analysis of DBPs, the reproducibility of the YES assay was assessed by measuring the dose-response of the yeast cells to duplicate samples of selected steroid hormones and 4-NP diluted over three orders of magnitude. Table 3.3 gives the EC_{50} readings for E2 taken during 7 independent experiments with a respective mean and standard deviation value of 2.47×10^{-10} M and 2.17×10^{-11} M. The relative standard deviation (RSD) of these E2 EC_{50} values was 8.76%. Taking the RSD into consideration gives EC_{50} values in the concentration range of 2.26×10^{-10} M - 2.69×10^{-10} M, which falls within the same magnitude as those reported in the literature as shown in Table 3.1. These results show that the procedure used while employing the yeast assay was precise and as such, one can accept the estrogenic activity result of a DBP such as iodoacetic acid that was assayed once.

The DBPs selected for this study were chosen based on their occurrence in drinking water and possible adverse health effects associated with them based on reports from *in vivo*, *in vitro* and epidemiological studies. Very few studies have analyzed the estrogenic activity of DBPs (Cargouet et al., 2007; Itoh et al., 2000; Nishihara et al., 2000) and a comparative database among a series of *in vitro* bioassays is lacking. The results presented in this study represent one of the few datasets available for the estrogenic activity of DBPs**.** Predicting their estrogenic activity by using their chemical structures would rule out estrogen receptor-mediated activity since most of them lacked the phenolic ring needed for binding to the hERα. All DBPs except iodoacetic acid were analyzed twice, with duplicate results shown in Appendix D. Analyzing DBPs in the absence of the yeast cells at non cytotoxic concentrations was a measure taken to rule out any possibility of false positive results due to their reaction with the assay medium. This would have generated o-nitrophenol, a yellow product that is formed from the reaction of β-galactosidase with o-NPG. When formed in the presence of yeast cells, its concentration, measured as a function of its absorbance at 450 nm, is correlated to the estrogenic activity of the tested chemical.

Interaction of hydroxyl groups on the steroid hormone (E2) with amino acids and a water molecule in the estrogen receptor

Some of the DBPs analyzed showing estrogenic activity in this study did not possess a phenolic ring in spite of the assumption, drawn from previous studies, that this was a pre-requisite for the chemical's ability to bind to the estrogen receptor (Fang et al., 2001; Sumpter & Routledge 1997; Blair et al., 2000). The steroid hormone (E2) has a phenolic ring and a hydroxyl group at position C-17 on its structure and these have unique functions when binding to the estrogen receptor. Recognition of E2 by the estrogen receptor is achieved by

the hydrogen bond formed by the phenolic hydroxyl group on the A–ring to the carboxylate of Glutamine 353, the guanidinium group of Arginine 394, and a water molecule on the estrogen receptor. The hydroxyl group at position C-17 of the D-ring forms a single hydrogen bond with Histidine 524 while the remainder of the E2 molecule is in hydrophobic contact with the other amino acids surrounding it (Brzozowski et al. 1997). The estrogen-receptor complex formed at this point is able to activate a gene which ultimately generates protein that has numerous functions in the body. Although the hydroxyl groups of E2 are mainly involved in interaction with amino acids, other unoccupied pockets of the estrogen receptor can accept other hydrophobic groups (Kuiper et al. 1998).

Observed estrogenic activity of haloacetic acids, haloacetonitriles, mucochloroc acid, and 2-bromoacetamide

Activation of the genomic pathway in the yeast cells by some of the DBPs assayed during this study might possibly be due to the fact that some of them such as mucochloric acid, and 2-bromoacetamide have a hydroxyl group and/or amine group on their structures, respectively. It is possible that these functionalities have the ability to serve as hydrogen bond donors that mimic that on the A-ring of E2, thereby interacting with certain amino acids of the estrogen receptor binding site (Brzozowski et al. 1997), especially when the DBPs are at a very high concentration such as those used in this study. Another possible explanation for the observed estrogenic activity triggered by binding of the DBP to the estrogen receptor could be that it is able to fill the unoccupied pockets of the estrogen receptor at higher concentrations. Such an action could possibly be mediated by the presence of chlorine, bromine or iodine atom as observed with the haloacetic acids analyzed during this study. This would explain why dibromoacetonitrile and dichloroacetonitrile showed estrogenic

activity despite lacking hydroxyl groups. The halogen on these haloacetonitriles was probably responsible for the interaction of the DBP with the receptor binding pocket positions where the substituent of E2 at position 7 α, 11 β, and 17 α would normally fit on the estrogen receptor. This has been stated as the reason why diethylstilbestrol (DES) and EE2 has a higher relative binding affinity to the estrogen receptor when compared to E2 (Fang et al., 2001).

Explanation for chloral hydrate's lack of estrogenic activity in the concentration range tested during this study

Chloral hydrate showed no estrogenic activity in the concentration range up to 1.51 x 10^{-5} M tested despite having functional groups (i.e. OH and Cl) that contributed to a positive response in other tested DBPs. The lack of estrogenic activity by this DBP might be due to the position of the hydroxyl group on its structure. It has been suggested that modification of the structure of E2 by the addition of a hydroxyl group at its position C-2, C-4, and C-16 causes a reduction in its relative binding affinity to the estrogen receptor (Fang et al., 2001), and this reason explains why 2- and 4-hydroxyestradiol are not as estrogenic as E2. It is possible that the close proximity/positioning of the hydroxyl groups on these modified E2 steroids and on chloral hydrate affects the normal interaction of a single hydroxyl needed with the amino acids and water molecule on the estrogen receptor.

Chlorophenols: Role of halogen and hydroxyl in binding to the hERα in the yeast cells

2,4-dichlorophenol (ranked 7th with an EC_{10} of 9.21 x 10^{-5} M) and 2-chlorophenol (ranked 9th with an EC_{10} of 8.84 x 10^{-4} M) both showed estrogenic activity and this can be attributed to their hydroxyl residues binding to the estrogen receptor of the yeast cells

(Michalowicz & Duda 2007). These phenolic compounds ranked lower than the haloacetonitriles and haloacetic acids analyzed during this study and, therefore, warrant further study to explain the mechanism behind such an observation. It would be expected that 2,4,6-trichlorophenol would be more estrogenic than 2-chlorophenol and 2,4-dichlorophenol since it has more chlorine atoms but it showed no activity. The increased number of chlorine atoms on 2,4,6-trichlorophenol possibly resulted in its reduced diffusion or inability to cross the yeast cell wall. The significance of this is that fewer molecules of 2,4,6-trichlorophenol probably reached the estrogen receptor and resulted in no binding action and therefore, no estrogenic activity. Another explanation could be that the chemical has a very low relative binding affinity to the estrogen receptor that could not be measured by the yeast cells employed during this study. A study showed that phenolic compounds such as 2,4-dibromophenol and 2,4,6-tribromophenol have a low relative binding affinity to the estrogen receptor and even displayed anti-estrogenic activity by reducing cell growth when co-incubated with E2 in a cell proliferation assay (Olsen et al., 2002). Another study showed 2,4,6-trichlorophenol as an anti-estrogenic compound that reduced the binding affinity of E2 to the rainbow trout estrogen receptor (Jobling et al., 1995). The extra chlorine atom on 2,4,6-trichlorophenol probably hinders its phenol hydroxyl group, thereby reducing its ability to form a hydrogen bond with an amino acid on the estrogen receptor (Schultz et al., 2003). It is also possible that the extra chlorine atom on 2,4,6-trichlorophenol may possibly withdraw electrons from its phenol hydroxyl-group, thereby preventing its interaction or causing it to have a low relative binding affinity to the estrogen receptor of the yeast cells (Olsen et al., 2002).

Differences in DBPs cytotoxic activity to the yeast cells and CHO mammalian cells

Results from this study showed that the yeast cells have a higher tolerance for the cytotoxic activity of DBPs such as 2-bromoacetamide and iodoacetic acid when compared to CHO mammalian cells. The difference in cytotoxic activity in these cells has to do mainly with the role of metabolism that occurs in some *in vitro* mammalian cell lines. Glutathione is a tripeptide found in cells and is mainly used during phase II reactions, which involves conjugation with a metabolite formed during phase I reaction. Conjugation of glutathione with a metabolite should form a product that is soluble in water and easily excreted from the body (Timbrell 1991). A study using mammalian CHO cells have suggested that a DBP such as iodoacetic is a soft electrophile and has a preference for reacting with soft nucleophiles such as thiol groups of cysteinyl residues in proteins and gluthatione (Plewa et al., 2004). At high concentrations used during *in vitro* studies, iodoacetic acid and its conjugated metabolites can deplete the cellular concentration of glutathione; thereby making the cell susceptible to cytotoxicity. Another study also highlighted the importance of glutathione in cells; whereby this tripeptide would detoxify DBPs such as dichloro- and dibromoacetonitrile (Plewa et al., 2007). Detoxification of these dihaloacetonitriles is only possible if both halogens are displaced after the DBP undergoes conjugation with glutathione. At high concentrations used during *in vitro* studies, displacement of a single halogen leads to formation of a highly reactive metabolite (α-halothioether) which can induce cytototoxicity. The process of conjugation of glutathione with DBPs that leads to formation of highly reactive intermediate metabolites that causes cell death/inhibition in CHO mammalian cells is probably absent in the yeast cells, and this explains their higher cytotoxic tolerance for DBPs.

DBPs concentration recommended for future studies using mammalian cell lines

Results from this study showed that dibromoacetic acid displayed almost a full dose response curve when analyzed in the concentration range of 6.73×10^{-5} - 4.64×10^{-4} M and no cytotoxic activity was observed even at the highest concentration assayed. According to the Plewa et al. (2002) study, dibromoacetic at 5.00×10^{-4} M caused 50% CHO cell density reduction; therefore, concentrations above this would cause an even higher percentage reduction. The cytotoxic activity in the Plewa et al. (2002) study suggests that analysis of this DBP for estrogenic activity might possibly have to be carried out at concentrations below 1.00×10^{-5} M where less or no cytotoxicity would be experienced in mammalian cells. Such lower concentrations would be a good starting point for future studies that analyze these DBPs for estrogenic activity, while effectively monitoring any evidence of cytotoxicity.

Applicability of the YES assay in screening DBPs and chemicals found in drinking water

The results from this study show that the yeast cells can be used as a screening tool to determine the estrogenic activity of DBPs despite significant differences in cytotoxic activity when compared to mammalian CHO cells. The yeast cell's cytotoxic resistance to DBPs at higher concentrations is an advantage in the sense that mammalian cells can't detect estrogenic activity at these concentrations due to their cell growth inhibition and death. Despite the fact that humans would not be exposed to DBPs at such high concentrations, the results from this study show that these compounds have the ability to activate the genomic pathway despite lacking some of the structural features of confirmed EDCs. This observation shows that rather than predicting the estrogenic activity of DBPs and other compounds by using their structural features, a quick and inexpensive screening tool such as the YES assay

can be used as employed during this study. Results from such quick screenings can then be used to decide which DBPs would be subject to further analysis by using *in vitro* assays employing mammalian cell lines. Another benefit of the YES assay is that the yeast cells are transfected with the human estrogen receptor (hERα). The absence of other estrogen receptors eliminates any possible interaction that would normally occur in mammalian cells that contain receptors for various steroids, peptide hormones and growth factors (Sumpter & Routledge 1996). A DBP such as iodo-, dibromo- and trichloroacetic acid that showed an estrogenic response when analyzed with the YES assay during this study can be said to have activated the genomic pathway employed in the yeast cells through its interaction with the hERα.

5.0 CONCLUSIONS

5.1 Implications of Results

The DBPs currently regulated in the U.S., are THM4, HAA5, bromate and chlorite, and DWTPs often choose to switch to the use of alternate disinfectants in order to meet the MCL set by the regulations for each DBP or DBP grouping. These alternate disinfectants also generate DBPs some of which have been shown to be more carcinogenic and cytotoxic than their chlorinated counterparts (Plewa et al., 2002, 2007, 2008; Kargalioglu et al., 2002). More than 600 DBPs have being identified at the present time with limited studies on their occurrence levels and health effects. Concerns have been raised about the ability of DBPs to influence the endocrine system by their possible estrogenic activity yet very limited data is available to refute or place more emphasis on this concern. It would be intuitive to suggest that most DBPs have little or no affinity to the estrogen receptor because of the absence of a phenolic ring. Yet natural organic matter which is the most predominant precursor of DBPs contains many aromatic substructures and it is likely that some of these remain intact after disinfection. Results presented in this thesis identified estrogenic activity using *in vitro* yeast-based assays in some chlorine-, bromine-, and iodine-containing DBPs albeit at much higher concentrations than their occurrence levels in drinking waters. This shows the value of using *in vitro* assays to screen for possible estrogenic activity of DBPs rather than depend on prediction using their chemical structures.

The DBPs analyzed during this study included some of those currently regulated, such as dibromoacetic and trichloroacetic acid, as well as those which are unregulated such as mucochloric acid, iodoacetic acid, and 2-bromoacetamide. Other phenolic compounds such as 2,4-dichlorophenol, 2,4,6-trichlorophenol and 2-chlorophenol were also analyzed because they can occur as byproducts of anthropogenic chemicals such as herbicides found in drinking water sources although their concentrations in drinking water are reported at less than 1 μg/L (Michalowicz 2005, Sithole & Williams 1986). Dichloroacetonitrile turned out to be the most estrogenic DBP among those tested in this study based on a relative rank order potency. Mucochloric acid ranked second, while 2-bromoacetamide ranked third. Dibromoacetonitrile ranked fourth while the regulated trichloroacetic and dibromoacetic acid ranked fifth and eighth respectively.

The fact that the unregulated DBPs analyzed in this study showed estrogenic activity in the yeast estrogen screen at lower concentrations than the regulated dibromo- and trichloracetic acids indicates the need for further study. The unregulated haloacetonitriles showing among the highest activity in this study can be formed at higher levels with chloramination than free chlorine while mucochloric acid that ranked second has been shown to steadily increase in concentration in the distribution system of a drinking water treatment plant that used free chlorine and chloramines (Weinberg et al., 2002). Current MCLs are not based on estrogenic activity and so DWTPs that switch from free chlorine to alternate disinfectants in order to meet these MCLs may not be adequately protecting the consumer. An important point to note is that the concentration of the DBPs showing estrogenic activity in this study are two or more orders of magnitude higher than their occurrence levels in drinking water as shown in Table 5.1.

Table 5.1 Comparison of DBP concentrations at their EC_{10} to their Occurrence Levels

DBP		Occurrence Levels		
	EC_{10} (μg/L)	75th Percentile (μg/L)	90th Percentile (μg/L)	Maximum Conc. (μg/L)
Dichloroacetonitrile	88	2.0	4.4	12.0
Mucochloric acid	534	N/R	N/R	0.71
2-bromoacetamide	2610	N/R	N/R	1.1
Dibromoacetonitrile	2620	N/R	2.3	2.0
Trichloroacetic acid	7860	N/R	26	N/R
Iodoacetic acid	20800	0.116	N/R	N/R
Dibromoacetic acid	27500	N/R	3.5	N/R

Cited studies are Weinberg et al., 2002 and 2011; #Krasner et al., 2006; @McGuire et al., 2002.
N/R: Not reported in occurrence study.

Consumers would not be exposed to the EC_{10} concentrations of the DBPs shown in Table 5.1 as long as water utilities are complying with the DBP regulations. Besides compliance by water utilities, metabolism and excretion of these DBPs by the body acts as a form of defense that reduces their concentration or eliminates them. It would, therefore, seem logical to conclude that humans are not at risk of exposure to levels of DBPs that cause estrogenic activity through consumption of drinking water but this is not so clear cut. This research only addressed the estrogenic activity of DBPs acting alone as single compounds and did not study the effect of the species in mixtures or even in the matrix of drinking water chemicals which includes residual disinfectants and natural organic matter, among others.

5.2 Proposed Future Research Efforts

It is necessary to address binary and ternary mixtures of DBPs to determine if their estrogenic activity becomes enhanced or reduced. Such mixture experiments should be designed in a manner that the estrogenic activity of a DBP does not significantly influence the activity of the mixture. For instance, a binary mixture experiment containing mucochloric acid and iodoacetic acid should have the former at a lower concentration since it showed

estrogenic activity at a much lower concentration than the latter in this study. Results from such mixture experiments can allow predictions to be made about possible interactions between different DBP classes especially since all identified 600 cannot be analyzed as single compounds or mixtures. Based on the results obtained from this study, dichloroacetonitrile showed more estrogenic activity at a lower concentration of 88 μg/L when compared to the other DBPs and phenolic compounds. Future research effort should also focus on analyzing this DBP since it is not currently regulated by the U.S. EPA. Trichloroacetic acid was more estrogenic than dibromo- and iodoacetic acid. This observation possibly suggesting that the chlorine-containing DBPs appear more estrogenic than their bromine- and iodine-containing counterparts perhaps indicates that the presence of chlorine atoms enhances binding to the estrogen receptor on yeast cells when compared to bromine and iodine. Future research efforts need to be directed at analyzing DBPs such as 2-bromoacetamide, iodoacetamide, and chloroacetamide simultaneously in order to determine if estrogenic activity decreases as their electronegatively increases. Such future research efforts should also employ *in vitro* assays using mammalian cell lines in order to clearly define if humans are at any risk due to exposure to these compounds in drinking water. Results using sensitive mammalian cell lines would be a better representation of how humans might possibly be affected by the estrogenic activity of DBPs but this study illustrated that with the appropriate quality control, the yeast estrogen screen can help with an initial rank ordering of DBPs for further evaluation by the more complex *in vitro* assays.

Future research should also focus on analyzing whole or simulated drinking water samples (that would better represent the matrix of human exposure to DBPs) by using *in vitro* mammalian cell lines. Various laboratory approaches to generate simulated drinking

waters at enhanced DBP levels can be employed. One approach involves the use of an automated pump-operated reverse osmosis (RO) system that has a membrane surface which can concentrate a large volume of source water from a water treatment plant (Speth et al., 2008; Simmons et al., 2002; Miltner et al., 2008; Richardson et al., 2008). Such an approach would also require chemical analysis in order to determine the amount of DBP that would be spiked into the final concentrate due to losses during the concentration effort. Chemical analysis for known DBPs needs to be performed after the concentration effort in order to ensure that their distribution matches those in the finished drinking water from the water treatment plant. Although such an approach would generate simulated drinking water, it normally requires a large volume of water and can be time consuming. Another approach that has been used for analysis of raw water and chlorination by products of EDCs (Beck et al., 2006; Leusch et al., 2010; Hu et al., 2003; Liu et al. 2005) involves disinfecting source water by using chlorine demand or target residual approaches before concentrating the water on a solid phase surface with the aim of retaining DBPs that can be eluted into a small volume of solvent. This approach is not as time consuming as the RO system but the blow down procedure of the solid phase extracts' eluent by a gentle stream of nitrogen can cause volatile DBPs such as the trihalomethanes with high vapor pressures to be lost. Solvents used during the concentration procedure have to be those that do not contribute to the estrogenic activity of the water samples; therefore, negative controls should be concentrated simultaneously during each experiment. Regardless of the approach used, it is possible that the final concentrate's extract may contain a slightly different distribution of DBPs and one cannot conclude that the observed estrogenic activity from the *in vitro* assay used is entirely due to

DBPs since other contaminants of concern would most likely be found in the concentrated drinking water.

APPENDIX A: Yeast Estrogen Screen (YES) Assay Protocol

This protocol is modified from the work of Routledge and Sumpter (1996) and Chen et al., (2007). The yeast strain (*Saccharomyces cerevisiae*) used for this assay was provided under agreement with Professor J.P Sumpter at Brunel University, UK. This protocol was written by Paul Ebohon at the University of North Carolina at Chapel Hill on January 4, 2011 and modified on July 9, 2011.

Part 1: Materials needed

96 well plate reader* (Molecular Devices, EMAX; Sunnyvale, California)

Centrifuge* (International Equipment Company; Needham Heights, Massachusetts)

Shaker table** (Barnstead International; Dubuque, Iowa)

Incubator** (Fisher Scientific; Dubuque, Iowa)

Weighting scale (Sartorius; Goettingen, Germany)

Stirrer (Barnstead/Thermolyne; Dubuque, Iowa)

Disposable filter sterilization flasks (Corning Incorporated; Corning, New York)

Disposable (100 x 15 mm) sterile petri dishes (Fisher Scientific; Suwanee, Georgia)

Disposable 96 well flat bottom microplates (Greiner-Bio-One; Frickenhausen, Germany)

Disposable 96 deep well 1 mL plates (Fisher Scientific; Suwanee, Georgia)

Falcon tube (Becton Dickinson Labware; Franklin Lakes, New Jersey)

Plate sealing film (Denville Scientific; Metuchen, New Jersey)

Disposable V shaped wells for multichannel pipetting (USA Scientific; Ocala, Florida)

Vortex mixer (Barnstead/Thermolyne; Dubuque, Iowa)

Original potable pipette aid (Drummond Scientific; Broomall, Pennsylvania)

50 – 300 µL 8 multichannel pipettor: 50 – 300 µL (Thermo Labsystems; Vantaa, Finland)

100 - 1000 µL pipettor (Fisher Scientific; Dubuque, Iowa)

Disposable (1 – 250 µL) pipette tips (Fisher Scientific; Suwanee, Georgia)

20-100 µL pipettor (Pipetman; Middleton, Wisconsin)

Weighing paper: 152 x 152 mm (Fisherbrand; Suwanee, Georgia)

1 L reusable media/solution glass bottles (Corning Incorporated; Corning, New York)

250 mL screw cap Erlenmeyer glass flask (Kimble Chase Kontes; Vineland, New Jersey)

1, 5, 10 and 25 mL plastic disposable pipettes (Fisher Scientific; Raleigh, North Carolina)

*Located in room *2104 and **1213.

Part 2: Chemicals

Chemical	CAS #	Brand/Source used by Weinberg lab
20% Sodium dodecyl sulfate	151-21-3	Fisher Scientific; Fair Lawn, NJ
2-mercaptoethanol (βME)	60-24-2	Acros Organics; New Jersey, NJ
Adenine sulfate	321-30-2	Acros Organics; New Jersey, NJ
Ammonium sulfate	7783-20-2	Mallinckrodt; Paris, KY
[a]$Na_2HPO4 * 7H_2O$	7782-85-6	Mallinckrodt; Paris, KY
Bacto agar	2014-03-31	BD; Sparks, MD
Bacto peptone	2012-04-22	BD; Sparks, MD
[b]$NaH_2PO4 * H_2O$	10049-21-5	Mallinckrodt; Paris, KY
Casamino acids	65072-00-6	Fisher Scientific; Fair Lawn, NJ
[c]$MgSO_4 * 7H_2O$	10034-99-8	Acros Organics; New Jersey, NJ
[d]$CuSO_4 * 5H_2O$	7758-99-8	EM Science; Gibbstown, NJ
Dextrose anhydrous	50-99-7	Fisher Scientific; Fair Lawn, NJ
Difco yeast nitrogen base	2014-11-30	BD; Sparks, MD
[e]o-NPG	369-07-3	Research Organics; Cleveland, OH
Potassium chloride	7447-40-7	Fisher Scientific; Fair Lawn, NJ
Potassium hydroxide	1310-58-3	Sigma-Aldrich; St.Louis, MO
Sodium carbonate	497-19-8	Mallinckrodt; Paris, KY
Sucrose	57-50-1	Fisher Scientific; Fair Lawn, NJ

[a]$Na_2HPO4 * 7H_2O$: Sodium hydrogen phosphate heptahydrate.
[b]$NaH_2PO4 * H_2O$: Sodium dihydrogen phosphate monohydrate.
[c]$MgSO_4 * 7H_2O$: Magnesium sulfate septahydrate.
[d]$CuSO_4 * 5H_2O$: Cupric sulfate pentahydrate.
[e]o-NPG: o-Nitrophenyl-β-D-galactopyranoside: This compound is shipped on ice and has to be stored in the freezer immediately upon receipt.

Part 3: Media and Buffer Solution Recipes

All solutions should be made in sterilized bottles or vials with laboratory grade water (LGW) and stored at room temperature unless otherwise stated in this SOP. Solutions can be used for

as long as 6 months and should be discarded in the event of a visible color change or visible turbidity.

Liquid Ura-Trp media for yeast cells growth (500 mL):

- Add 300 mL of LGW and a magnetic stir bar into an autoclaved 1 L sterile reusable media/solution glass bottle.
- 3.35 g of Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate
- 2.5 g of ammonium sulfate
- 2.5 mL of adenine sulfate (4 mg/mL)
- 10 g of anhydrous dextrose
- 2.5 g of casamino acids
- Add 200 mL of LGW into the bottle containing the compounds above and place on a stirrer.
- Stir the contents in the sterile reusable media/solution glass bottle until they are dissolved.
- Filter sterilize using a disposable filter sterilization flask and transfer into another autoclaved 1 L sterile reusable media/solution glass bottle before storing at room temperature.

Ura-Trp Solid media for yeast cell propagation (500 mL):

- Add 10 g of bacto agar into an autoclaved 1 L sterile reusable media/solution glass bottle containing 500 mL liquid Ura-Trp media and a magnetic stir bar.
- Stir the contents of the bottle for approximately 10 minutes.
- Autoclave using the liquid cycle and limit sterilization time to 15 minutes because a longer one will degrade dextrose in the media.
- Place autoclaved container under hood and allow cooling until it can be handled without difficulty. Do not shake the content of the autoclaved bottle at this point because this would cause bubbles to be present in the media.
- Pour 15-20 mL of media into 25 disposable sterile petri dishes.

- Rinse the 1 L sterile reusable media/solution bottle with LGW and place in a bin containing glassware that needs to be cleaned according to laboratory procedure.

- Let stand at room temperature to harden.

- Place covered petri dishes in dated Ziploc bags and store at 4°C.

<u>YPS Media used to make the diluted yeast solution (500 mL)</u>:

- Use the same technique described for liquid Ura-Trp media.

- 5 g YNB

- 2.5 g Peptone

- 50 g Sucrose

- Filter sterilize using a disposable filter sterilization flask and transfer into an autoclaved 1 L sterile reusable media/solution glass bottle before storing at room temperature.

<u>Z Buffer solution for the o-NPG colorimetric substrate (1 L)</u>:

- Add 500 mL of LGW and a magnetic stir bar into an autoclaved 1 L sterile reusable media/solution glass bottle.

- 16.1 g $Na_2HPO_4 * 7\ H_2O$ (60 mM final)

- 5.5 g $NaH_2PO_4 * H_2O$ (40 mM final)

- 0.75 g KCl (10 mM final)

- 0.246 g $MgSO_4 * 7\ H_2O$ (1 mM final)

- Add another 500 mL of LGW into the bottle containing the compounds above and place on a stirrer.

- Stir the contents in the reusable media/solution bottle until they are dissolved.

- Adjust to pH 7 by using a 2 M Potassium Hydroxide (KOH) solution.

- Filter sterilize using a disposable filter sterilization flask and store at room temperature. Immediately before using, add 135 µL of β-mercaptoethanol (βME) per 50 mL of Z-buffer solution .βME cannot be added in advance because it becomes oxidized and loses its potency over time.

- The o-NPG is added after βME (See Instructions for Day 5).

10% Sodium dodecyl sulfate (SDS) used to denature proteins prior to colorimetric measurement:

NOTE: Make in small batches because it will lose its potency after about a month.

- Transfer 5 mL of LGW into a 250 mL screw cap Erlenmeyer glass flask.
- Transfer 5 mL of 20% SDS into the bottle containing LGW and swirl contents.
- Label bottle as 10% SDS and include initials, date and time of preparation.

1M sodium carbonate solution that stops the reaction of β-galactosidase with o-NPG by shifting the reaction mixture to pH 11:

- Dissolve 59.5 g sodium carbonate in 500 mL of LGW by using the technique described for liquid Ura-Trp media.
- Filter sterilize using a disposable filter sterilization flask and store at room temperature.

$CuSO_4$ solution that helps to increase expression of the estrogen receptor on the yeast:

- Transfer 100 mL of LGW into a 250 mL volumetric flask.
- Transfer 0.122g $CuSO_4 * 5H_20$ into the volumetric flask above.
- Fill the flask above with LGW until you reach the 250 mL line.
- Invert 3 times and filter sterilize using a disposable filter sterilization flask and store at room temperature.

10% Ethanol (EtOH) used for serial dilution of the samples and the E2 standard (See Appendix C for preparation of DBP stock and working solutions):

NOTE: In order to avoid possible volatilization of the EtOH from LGW, make this solution right before performing the assay.

- Dissolve 1 mL of ethanol in a 250 mL screw cap Erlenmeyer glass flask containing 9 mL of LGW.

17-β-estradiol (E2) stock solutions (in 100% ethanol)[1]:

- Make a 1E-2M E2 stock solution: 27.38 mg of E2 into 10 mL ethanol.
- Prepare 1E-4M E2 stock solution by spiking 100 uL of 1E-2M E2 stock solution into 10 mL ethanol.
- Prepare 1E-6M E2 stock solution by spiking 100 uL of 1E-4M E2 stock solution into 10 mL ethanol.
- Store all stock solutions at -20°C.

5E-8M 17-β-estradiol (E2) working standard solution made in 10% ethanol:

- Pipette 9 mL of LGW into a clean sterilized amber vial.
- Add 1 mL of ethanol followed by 1 mL of 1E-6M E2 stock solution into the 9 mL LGW.
- Add an additional 9 mL LGW into the amber vial.
- Cap and invert multiple times in order to mix the solution properly.
- Store at 4°C.

NOTE: This E2 working standard solution can be used for at least two weeks; however, it is recommended to make it fresh before each assay.

Part 4: Yeast Cell Propagation and Assay Procedure:

1. Yeast cell propagation

I. Cell growth on solid Ura-Trp media: A petri dish containing viable yeast colonies was provided by North Carolina State University and this dish was subsequently used for streaking the next yeast generations.

1) Using a flame looped sterile wand, streak a single colony of yeast from a previous dish containing viable yeast colonies onto an Ura-Trp solid media dish. Seal the plate with parafilm and incubate at 30°C. After 60 - 72 hours (when individual colonies have reached 1-2 mm in diameter), the dish should be removed from the incubator and stored at 4°C.

1. Always store E2 stock and working standard solutions in amber glass vials. Plastic vials such as falcon tubes may leach and alter the estrogenic activity of the E2 standard.

NOTE: Dish containing yeast colonies can be stored at 4°C for 2 months; however, it is a good practice to streak fresh plates at least every month in order to keep the yeast colonies viable.

2. Assay Procedure

NOTE: Slightly different procedures must be followed depending on whether samples are prepared in 10 or 100% ethanol and other solvents. See details below based on what solvents your samples are prepared in.

DAY 1

II. NOTE: If your samples are prepared in 100% EtOH[2], you will need to prepare your 96 well plates today in order to allow the solvent to evaporate overnight. *This assay procedure assumes duplicate analysis for each sample with E2 being used as positive control, and 10% ethanol as a negative control on every plate. A separate plate containing the samples, E2 and negative control should also be assayed simultaneously with the assay media containing no yeast cells in order to determine if the samples react with o-NPG which would generate false positive results. When analyzing DBPs, do not use this step because they do not evaporate completely after 24 hours.*

1) Add 200 μL of 5E-8M E2 standard (in 100% EtOH) to the first column of rows A and B (See Figure 1 below).

2) Add 200 μL of sample (in 100% EtOH) in duplicate to row C-H. A total of 3 samples can be run on each plate (See Figure 1 below).

3) Place the plates in the fume hood for 24 hours to completely evaporate solvent in the wells.

NOTE: Cover plates loosely with Kim wipes to prevent dust from falling in.

III. Grow yeast cells in liquid Ura-Trp media[3]:

1) Aliquot 7 mL of liquid Ura-Trp media into a 50 mL sterile falcon tube.

2) Using a sterile wand, pick up one independent yeast colony from a solid Ura-Trp dish containing viable yeast colonies and transfer it to the liquid media in the falcon

2. This assay has been optimized for samples dissolved in ethanol; however, samples dissolved in methanol have also been shown to work relatively well. If samples in methanol are used, an E2 standard in methanol should also be used.

3. Although this procedure is carried out on the bench top, sterile techniques such as flaming the mouth of media bottles before and after media withdrawal should be used.

tube.

3) Incubate the falcon tube containing the yeast colony at 30°C on a shaker table at ~200 rpm for 24 hours.

DAY 2

IV. Determination of yeast cell density and its dilution using YPS media

1) Vortex the falcon tube containing the 24 hour yeast suspension culture by using a vortex mixer for approximately 30 seconds. Ensure that the 96 well plate reader is turned on at least 10 minutes prior to use.

2) Using a 20-100 µL pipettor, place 100 µL of the yeast suspension above in wells A1, A2 and A3 of a 96-well flat bottom microplate before adding 100 µL of YPS media in wells B1, B2, and B3 on the same plate. Read the plate at an absorbance of 600 nm in the plate reader.

3) Calculate the total volume of yeast cell solution that is used for each set of experiments:

a) Subtract Abs_{600YPS} from $Abs_{600Yeast}$[4]

b) Solve for x: $(Abs_{600Yeast} - Abs_{600YPS})$ (x µL) = (0.07) (100 µL). 0.07 is the target corrected absorbance reading for the diluted yeast solution measured at an absorbance of 600 nm.

c) Solve for y: (x µL)/(100µL) = (y mL)/(33 mL)

d) y mL of yeast + (33-y) mL of YPS = dilution of the yeast suspension needed for the assay.

e) Make the yeast dilution calculated above. In order to ensure that there is enough yeast solution; make 33 mL of diluted yeast suspension per 96 deep well 1 mL plate.

f) Check your yeast dilution to make sure that the Abs_{600} falls in the range of 0.06 and 0.08 (Once again, subtract Abs_{600YPS} to account for background).

g) Add 100 µL of $CuSO_4$ solution per 10 mL to the diluted yeast solution and vortex to mix.

4. After 24 hours, the yeast suspension in liquid Ura-Trp should have an Abs_{600} between 0.5 and 0.9. The YPS media blank should have an Abs_{600} similar to that of an empty well and is typically around 0.03.

DAY 2

V. Prepare 96 deep well 1 mL plates: If your samples were prepared in 10% ethanol, ignore step 2 below.

NOTE: If your samples and E2 working standard solution were prepared in 100% EtOH, you should have completed this step on Day 1 in order to allow evaporation overnight.

1) For samples prepared in 10 and 100% EtOH: Using an 8 multichannel pipettor, add 100 µL of freshly prepared 10% ethanol to each well in columns 12-2 of a 96 deep well 1 mL plate.

2) For samples prepared only in 100% EtOH (See Day 1): Using an 8 multichannel pipettor, add 200 µL of 10% EtOH to each of the 8 wells in column 1 of the 96 deep well 1 mL plate. Aspirate each well thoroughly with the multichannel pipettor in order to resuspend the samples in the 10% EtOH.

3) For samples prepared in 10% EtOH: Add 200 μL of 5E-8M E2 working standard solution to the first column of rows A and B (Wells A1 and B1). Add 200 μL of samples (prepared in 10% EtOH) in duplicate to row C-H. A total of 3 different DBPs can be run on each plate if 11 points on the dose response curve are required with each duplicate (See Figure A.1 below).Using an 8 multichannel pipettor, dilute each column serially in 1:2 dilutions. (Transfer 100 µL of sample from column 1 to column 2; mix thoroughly by aspirating, then transfer 100 µL from column 2 to column 3. Continue the serial dilution across entire plate until you get to column 11. After mixing the contents of column 11, withdraw 100 uL that would be discarded as waste so that the column 12 wells containing the negative control as shown in the YES assay template (Figure A.1) has only 10% ethanol at this point.

→ Columns

	1	2	3	4	5	6	7	8	9	10	11	12
A	E2 Standard (Row A and duplicate in Row B, Columns 1-11)											Negative Control with 10% EtOH
B												
C	Sample 1 (Row C and duplicate in Row D, Columns 1-11)											
D												
E	Sample 2 (Row E and duplicate in Row F, Columns 1-11)											
F												
G	Sample 3 (Row G and duplicate in Row H, Columns 1-11)											
H												

Figure A.1 YES Assay Template

VII. Exposure of yeast cell to samples:

1) Vortex the falcon tube containing diluted yeast solution for approximately 30 seconds and pure its content into disposable V shaped well.

2) Using an 8 multichannel pipettor, add 300 µL of the diluted yeast solution to each well of the 96 deep well 1 mL plate containing the samples, E2 and negative control. Ensure that you perform this addition by starting from column 12 which contains the negative control. Ensure that each pipette has the same level of diluted yeast solution and no bubbles prior to placing in wells.

3) Cover plate with a plate sealing film and incubate for 3 days at 30°C while shaking at ~200 rpm. Ensure that you avoid opening and closing the closing the incubator until incubation period has elapsed.

DAY 5

VI. Measurement of optical densities (Endpoint):

1) Aliquot the amount of Z-buffer you will need into a 250 mL screw cap Erlenmeyer glass flask containing a magnetic stir bar. You will need about 50 mL of Z-buffer per assay plate. Add 135 µL of βME per 50 mL of Z-buffer and mix thoroughly by placing on a stirrer for approximately 5 minutes.

2) Prepare o-NPG assay buffer: NOTE: Use as soon as you remove from freezer and return immediately. Carefully transfer 42 mg of o-NPG weighed with a weighing paper into a 250 mL screw cap Erlenmeyer glass flask containing a magnetic stir bar before carefully adding 41 mL of the freshly-prepared Z-buffer + βME. Place the 250 mL screw cap Erlenmeyer glass flask on a stirrer and allow o-NPG to dissolve. This would take approximately 30 minutes. Once o-NPG is dissolved, add 1 mL 10% SDS and an additional 525 μL of βME. Mix the contents of this solution thoroughly.

3) Using an 8 multichannel pipettor, add 50 µL of Z-buffer (NOTE: Plain Z-buffer + βME and not the assay buffer) to each well of the 96 deep well 1 mL plate. Replace the plate sealing film on the plate if you observe any vapors or liquid on it. Mix the plate's contents at room temperature by shaking at ~300 rpm for 3-5 minutes on a shaker table.

4) Remove the plate sealing film and use a multichannel pipettor to add 400 µL of the o-NPG assay buffer to each well. NOTE: Ensure that no bubbles are present in the pipette tips and each has the same level of o-NPG assay buffer. Reseal the well with the same plate sealing film.

5) Incubate the plate at 30°C for 20 minutes while shaking at ~200 rpm.

6) Using an 8 multichannel pipettor, add 200 µL of 1 M sodium carbonate to each well to stop the reaction and reseal the 96 deep well 1 mL plate with its sealing film.

7) Centrifuge the plate at room temperature for 10 minutes at 3000 rpm in order to allow the yeast cells settle to the bottom of the 96 deep well 1 mL plate.

8) Using an 8 multichannel pipettor, withdraw 100 µL of clear supernatant from each well of the 96 deep well 1 mL plate and place on a fresh sterile 96 well flat bottom microplate. Ensure that you do not withdraw any yeast cell debris during this step. Avoid allowing bubbles in the 96 well flat bottom microplate because they will interfere with the plate reader's result.

9) Measure the absorbance of the contents of the fresh plate at 450 nm by using a plate reader. Export the 450 nm measurements into an excel spreadsheet and transfer the spreadsheet into a USB drive. Proceed to calculations in Part 5 and appendix B of this protocol). Once the data analysis is complete and you are sure that you don't need to re-measure the absorbance of the samples at 450 nm, proceed to step 10.

10) Using an 8 multichannel pipettor, thoroughly mix the contents of the 96 deep well 1 mL plate in step 8 so that the yeast cells are resuspended in the wells.

a) Using a multichannel pipettor, mix the contents of column 12 by aspirating each well about 10 to 15 times.

b) Transfer 100 µL of the contents of column 12 into another fresh sterile 96-well flat bottom microplate.

c) Push the top of multichannel pipettor's button so that the contents of the pipette tips are discarded on a Kim wipe. Continue this process until no bubbles are visible on the pipette tips. Repeat the process again for column 11 and work your way to column 1.

11) Measure the absorbance of the plate (in step 10) containing the resuspended yeast solution at 600 nm and place raw readings under template 4 that is set up exactly as template 2 under Part 5 of this protocol. Compare the sample absorbance at 600 nm to that of the negative control's average. Values that are significantly less than that of the negative control's average are taken as indication of cytotoxicity to the yeast cells due to the presence of the sample. Such cytotoxic concentrations are excluded from the data set used to plot the dose response curves for estrogenic activity.

Clean up:

1) All plates and disposable containers that have been inoculated with yeast cells should be autoclaved prior to disposal.

2) All glassware should be cleaned according to the glassware cleaning procedure instructions posted at room 1210B, wrapped with aluminum foil and stored in their appropriate cabinet.

3) Pour unused assay media, and chemicals into properly labeled disposal container and store appropriately according to laboratory procedure.

Part 5: Yeast Estrogen Screen Calculation Procedure to Determine EC_{50} for E2 Calibration Curve

For the calibration curves, you should have two rows (A & B) containing 11 concentrations of E2, ranging from a pre-dilution concentration of 5E-8 to a maximum dilution of 4.9E-11 M. Remember that you added 100 μL of the E2 standard to the wells before diluting it with 300 μL of yeast suspension; therefore, the actual final concentrations/concentration factors are the pre-dilution concentrations divided by 4. In your calculations E2 concentrations will range from 1.25E-8 to 1.22E-11 M.

Label an excel spreadsheet as shown below:

Template 1:

Column A: Labeled as compound with the corresponding samples listed as ran on the YES assay plate.
Column C: Labeled as absorbance at 450 nm

Column N: Labeled as negative control

Column P: Labeled as average of negative control

1) Transfer the raw absorbance readings into template 1 of the excel spreadsheet. The highest absorbance for E2 should be at cells C2 and C3.

2) Average the absorbance 450 nm (Abs_{450}) of the negative controls in order to get a single negative control value.

3) Leave some spaces after template 1 and set up template 2 (in the same format as template 1 but exclude column P) on the same page of the excel spreadsheet. Template 2 will contain your corrected absorbances for E2, and samples.

4) Template 2: For each well on the plate, subtract the single Abs_{450} of the negative control from Abs_{450} of sample. This value (Abs_{450} of sample – Average Abs_{450} of negative control) will simply be referred to as "Abs 1" and "Abs 2".

5) Leave some spaces after template 2 and set up template 3 (as shown below) on the same page of the excel spreadsheet in order to plot the dose response curve for E2 and samples.

Template 3: Columns may vary based on how you want your sheet set up**.**

Column A: Concentration.

Column B: Abs 1.

Column C: Abs 2.

You have 2 absorbances since each sample was run on 2 rows during the assay.

Column D: % Induction 1.

Column E: % Induction 2.

Column F: Top.

Column G: Bottom.

Column H: EC_{50}.

Column I: Lower 95^{th} % confidence interval for EC_{50}.

Column J: Upper 95^{th} % confidence interval for EC_{50}.

Column K: Hillslope.

Column L: EC_{10}.

6) Transfer the E2 concentrations into column A of template 3 with the highest concentration being on top.

7) Transfer the corrected absorbances at 450 nm into column B and C of template 3 and proceed to Appendix B.

APPENDIX B: Instructions for working with YES data in Graphpad Prism 4.03

1) Using the corrected absorbances in rows B and C; plot Abs 1 and Abs 2 as a function of the log of molar concentration of E2 using the Graphpad Prism 4.03 graphing program:

   - In Prism, select "XY" graph, and select "2" as the number of replicates.

   - In the data sheet, enter the E2 concentrations as the X values, and corresponding Abs as the Y values.

   - Go to the graph. Click "Analyze", "Data manipulations", select "Transform", and select "Transform X values X=Log(X)". This will log transform the X-axis.

2) Fit the data points to a sigmoidal dose-response curve:
   - Go to the graph of your E2 standard curve titled, "Transform of Data 1 graph".

   - Click "Analyze", select "Curves and Regressions", followed by "Nonlinear regression (curve fit). Click "OK". Select "Sigmoidal dose response (variable slope)" and click "OK". Label the X axis as the log of molar concentration and the Y axis as the corrected absorbance.

3) Find the Top (maximum) and Bottom (minimum) points of your standard curve.

   - Go to the Results section for your E2 standard curve.

   - You will see the "Top" and "Bottom" points listed.

4) Calculate percent induction for each E2 concentration, using the equation:

$$\%\ \text{Induction} = \frac{(\text{Abs} - \text{Bottom})}{(\text{Top} - \text{Bottom})} \times 100$$

5) Plot the percent induction as a function of log [E2] with GraphPad Prism:

   - On a new data sheet, enter the E2 concentrations as the X values, and corresponding percent inductions as the Y values.

   - Log transform the curve: Go to the graph, click "Analyze", select "Transforms", and select "X=Log(X)".
   - Fit a sigmoidal curve: Click "Analyze", select "Curves and Regressions", and select "Sigmoidal curve (variable slope)". On the "Constraints" tab, set "Top = "100" and "Bottom = "0".

6) Find the hillslope and EC_{50} under Results[5]. These numbers will be used to calculate the EEQ for the samples:

- Go to the Results section for the standard curve.
- You will see "Hillslope" and "EC_{50}" values listed.
- In order to calculate $EC_{10,}$ use the link below and you will require the Hillslope and EC_{50} from the Results generated by Graphpad prism software.
- http://graphpad.com/quickcalcs/Ecanything1.cfm

5. EC_{50} for the E2 standard curve varies from plate to plate but should typically fall within a range reported in the literature. If your EC_{50} value falls outside the reported range, this may indicate a problem with the assay so you may want to rerun your samples.

Calculation procedure to determine relative EEQ for samples without unknown concentrations

In this part, we treat each duplicate as a separate run. For environmental samples, we will be working with an estrogenic response as a function of Concentration Factor (CF). CF is defined as the degree to which the original sample has been concentrated. For example, if the initial environmental sample is concentrated from 100 mL to 1mL, it now has a CF of 100. Considering this sample is only ¼ of the final well volume, we must again divide the CF by 4 as we did to the [E2] in the calibration section. The final CF of the sample in column 1 is now only 25 instead of 100. If each well after this initial well is a 1:2 dilution of this, the CF will drop accordingly, to 12.5, 6.25, 3.125, etc. until a CF of 0.0122, assuming the same dilutions for all 11 wells.

1) Follow the same calculation procedure stated earlier (Step 1-6 listed under Appendix B).

2) Calculate the percent induction (as stated earlier) for each of the sample wells by using the same Top and Bottom values from the E2 standard curve.

3) Plot the sample % induction values.

- In a new data sheet, enter sample concentration factors as the X values, and the corresponding sample % inductions as the Y values.
- Log transform the curve: Go to the graph, click "Analyze", select "Transforms", and select "X=Log(X)".
- Fit a sigmoidal curve: Click "Analyze", select "Curves and Regressions" and select "Sigmoidal curve (variable slope)". On the "Constraints" tab, set the "Hillslope" equal to the Hillslope of the E2 standard curve. This is important, as it will allow you to compare your samples directly to the E2 standard curve.

4) Find the EC_{50} of the sample dose-response curves (referred to hereafter as the "CF_{50}"):

- Go to the "Results" section for your sample graph.
- You will see an EC_{50} listed for each of your samples.

5) Calculate EEQ for each sample using the equation: $EEQ = \frac{EC_{50}}{CF_{50}}$

6) As a measure of statistical accuracy, you may also wish to also record the 95% confidence interval around the CF_{50}. This can be found on the Results tab in Graphpad Prism 4.03.

APPENDIX C: Preparation of DBP Stock and Working Solutions

I. Technique for preparation of stock solution

a. Place a weighing dish on the balance of the weighing scale before tarring.

b. Ensure that the door to the balance is properly closed before weighing out your compound.

c. Using a 9'' disposable Pasteur pipette, transfer approximately 1 full pipette of ethanol into a 10 mL volumetric flask.

d. Cap the flask and invert 3 times before discarding the ethanol as waste.

e. Using a 9'' disposable Pasteur pipette, transfer approximately 2 pipettes full of ethanol into the 10 mL volumetric flask that was rinsed with ethanol.

f. Using a sterile spatula wrapped with PTFE tape, weigh the amount of the compound needed on the weighing dish. You don't need to have the exact calculated weight but get as close as possible or slightly above.

g. Record the weight to 4 decimal digits if the balance is accurate to this extent.

h. Cap the source chemical's container and move to a secure area under the hood where it would not be contaminated.

i. Carefully remove the weighing dish containing the compound from the weighing scale and use a 9'' disposable Pasteur pipette to transfer approximately 2 pipettes full of ethanol into the content of the dish.

j. Carefully transfer the contents of the dish into the 10 mL volumetric flask containing ethanol by pouring carefully.

k. Rinse the dish completely with ethanol by using a 9'' disposable Pasteur pipette and transfer the dish's content into the flask while ensuring that you take note of the 10 mL line.

l. Fill up the 10 mL volumetric flask to the line with ethanol, cap and invert 3 times before transferring into a clean 10 mL amber vial.

m. Calculate the actual concentration of the stock solution and record in lab notebook.

n. Label the amber vial containing the freshly prepared stock solution with its concentration, content, date, initials, and date prepared before storing in a freezer at -15°C.

o. Brush off scale gently and turn off before cleaning any glassware used for preparation of the stock solution.

II: Calculation showing how to prepare a stock solution of 2,4-dichlorophenol at 10000 mg/L in 10 mL ethanol

NOTE: 2,4-dichlorophenol is in a solid form so this procedure applies to other compounds in the same state.

1) The target concentration of this compound is 10000 mg/L. Multiply this concentration by 0.01 L of ethanol needed. This would give 100 mg, so you would need to transfer 0.1 g of 2, 4-dichlorophenol on the weighing dish (based on the technique described above) into a 10 mL volumetric flask containing ethanol.

2) Since it's not necessary to get the exact amount, 0.10296 g was used during this research and this figure was rounded to 0.10300 g.

3) The actual concentration of this compound in 10 mL ethanol is 10300 mg/L.

a) Convert 0.10300 g into mg to get 103 mg.

b) 103 mg of 2, 4-dichlorophenol is in 10 mL ethanol but we need this concentration in mg/L.

c) (103 mg) / (10 m L) * (1000 mL) / (1 L) = 10300 mg/L.

III: Preparation of a working standard solution of 2, 4-dichlorophenol at 278 mg/L in 10% ethanol (Solubility of 2, 4-dichlorophenol is 470 mg/L and this value was obtained from SciFinder for Academics, American Chemical Society, 2011).

NOTE: Before preparing any working standard solution, determine their solubility and vapor pressure by looking on SciFinder for Academics, American Chemical Society, 2011.

This working standard solution at 278 mg/L was prepared at a concentration that is below the solubility of this DBP in order to ensure its complete dissolution in 10% ethanol.

Vapor pressure is the pressure of the vapor that is formed above its liquid or solid and is constant under isothermal conditions. An increase in temperature would cause an increase in vapor pressure and chemicals with a high vapor pressure such as the trihalomethanes will be lost as vapor during the YES assay incubation period at 30°C. Ensure that the chemicals analyzed with the YES assay have a low vapor pressure.

1) Using a 9'' disposable Pasteur pipette, transfer approximately 1 full pipette of LGW into a 10 mL volumetric flask.

2) Cap the flask and invert 3 times before discarding the LGW as waste.

3) Using a 9'' disposable Pasteur pipette, transfer 2 pipettes full of LGW into the 10 mL volumetric flask that was rinsed with LGW.

4) Calculate how much volume of the stock concentration at 10300 mg/L would be transferred into the volumetric flask.

$C_1V_1 = C_2V_2$

C_1: Concentration of the stock solution at 10300 mg/L.

V_1: Volume that needs to be transferred from C_1 into V_2.

C_2: Desired final concentration of working standard solution at 278 mg/L.

V_2: Final volume of solvent which is 10 mL.

5) Based on the equation above, you would need to transfer 270 µL of the stock solution at 10300 mg/L into a final solvent volume of 10 mL.

6) Using a 1000 µL pipettor with an autoclaved pipette tip, carefully transfer 730 µL of ethanol into the 10 mL volumetric flask containing 2 pipettes full of LGW. Avoid touching the sides of the volumetric flask.

7) Using a 100 - 1000 µL pipettor with an autoclaved pipette tip, carefully transfer 270 µL of the stock solution at 10300mg/L into the 10 mL volumetric flask in step 6.

8) Using a 9'' disposable Pasteur pipette, fill the volumetric flask to the 10 mL line, cap and invert 3 times.

9) Transfer contents of the volumetric flask into an amber bottle and label appropriately before storing at 4°C.

NOTE: If V_1 or the volume of ethanol that needs to be transferred as shown in step 6 is between 50 and 250 µL, use a positive displacement digital micropipette attached to the appropriate glass microdispenser.

From the working standard solution example, follow steps 1 to 5. Assume that V_1 is 100 µL.

7) Adjust the positive displacement digital micropipette to the desired volume needed.

8) Clean the micropipette tip with methanol and dry with a Kim wipe.

9) Insert 100 – 200 µL glass microdispenser into the micropipette and tighten.

10) Carefully withdraw 100 µL of the stock solution and inject under liquid level in the volumetric flask containing 2 pipettes full of LGW, and 900 µL ethanol.

11) While still holding the button on top of the micropipette, swirl clockwise about 5 times before pulling it out of the volumetric flask.

12) Using a 9'' disposable Pasteur pipette, fill the volumetric flask to the 10 mL line, cap and invert 3 times.

13) Transfer contents of the volumetric flask into an amber bottle and label appropriately before storing at 4°C.

14) Remove the glass microdispenser from the micropipette and follow glassware cleaning procedure.

15) Rinse micropipette with methanol, dry with Kim wipe and store appropriately.

IV: Calculation showing how to prepare a stock solution of dibromoacetonitrile at 10000 mg/L in 10 mL ethanol (Information written on Material Safety Data Sheet and Container: Density of dibromoacetonitrile: 2.960g/mL; Purity: 95%)

NOTE: Dibromoacetonitrile is in a liquid form so this procedure applies to other compounds in the same state.

a) The target concentration of this compound is 10000 mg/L. Multiply this concentration by 0.01 L of ethanol needed. This would give 100 mg, so you would need to transfer 0.1 g of dibromoacetonitrile (based on the technique described above) into a 10 mL volumetric flask containing ethanol.

b) Spike 50 µL of dibromoacetonitrile into 10 mL ethanol. See calculation on how 50 µL was obtained.

$$0.1\text{g of compound} \frac{(1\text{ mL compound})}{(\text{Density of compound})} * \frac{(1\text{ solution})}{(0.95\text{ compound})} = 45.8\ \mu\text{L}$$

c) Actual concentration

$$50\ \mu\text{L} * \frac{(1000\text{ m L})}{(10^6\ \mu\text{L})} * \frac{(0.95\text{ compound})}{(1\text{ solution})} * \frac{(2.2960)}{(1\text{ m L compound})} = 0.10906\text{g}$$

Actual concentration in 10 mL ethanol is 10906 mg/L

APPENDIX D: Figures Showing Duplicate Results for DBPs and Phenolic Compounds

The figures below shows the duplicate dose-response curves for the DBPs and phenolic compounds analyzed during this study. Iodoacetic acid is the only DBP that was not analyzed twice but a relative standard deviation of 8.76% calculated for 7 independent positive control's EC_{50} shows a high precision used while employing the YES assay. Such a high precision made it possible to accept the estrogenic response for iodoacetic acid that was assayed once.

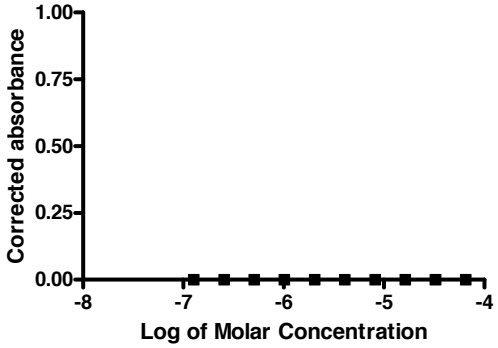


Figure D.1 Dose response curve for 2, 4, 6-trichlorophenol in the YES assay

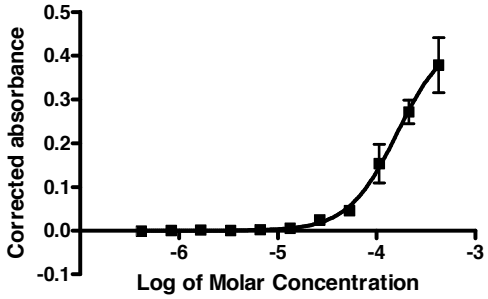


Figure D.2 Dose Response Curve for 2, 4-dichlorophenol in the YES assay

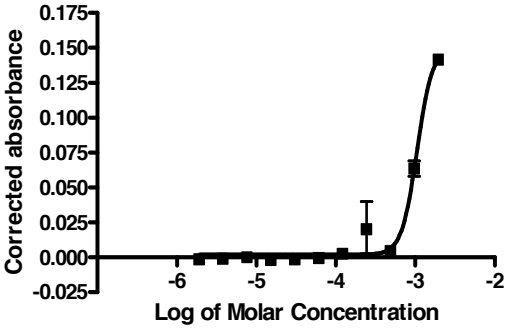


Figure D.3 Dose Response Curve for 2-chlorophenol in the YES assay

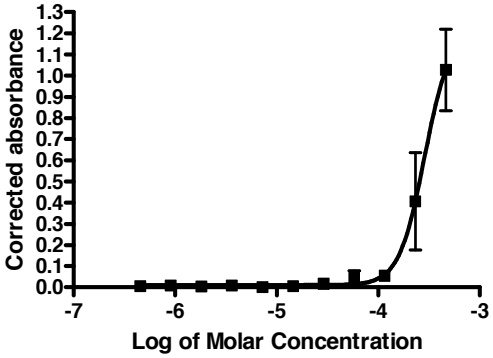


Figure D.4 Dose Response Curve for Dibromoacetic acid in the YES assay

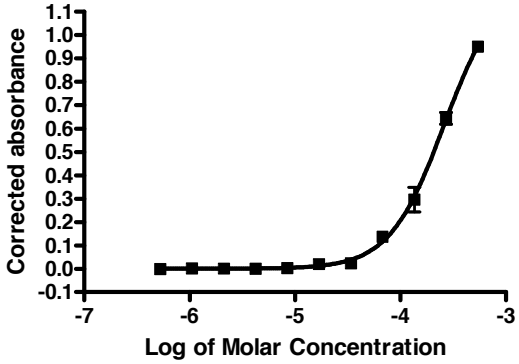


Figure D.5 Dose Response Curve for Trichloroacetic acid in the YES Assay

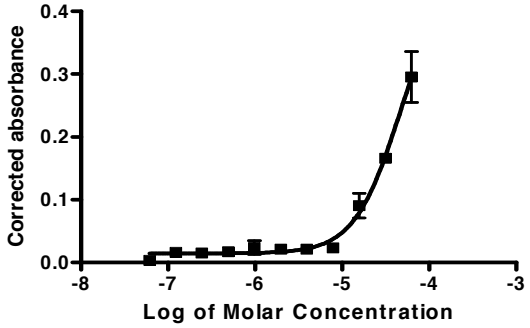


Figure D.6 Dose Response Curve for Dibromoacetonitrile in the YES Assay

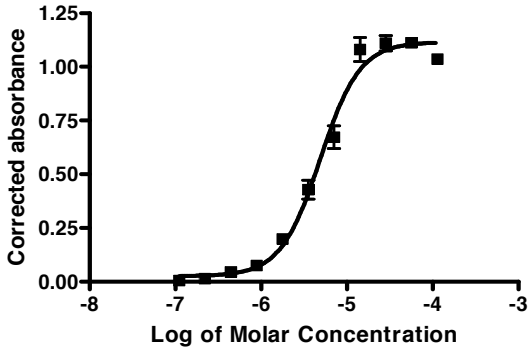


Figure D.7 Dose Response Curve for Dichloroacetonitrile in the YES Assay

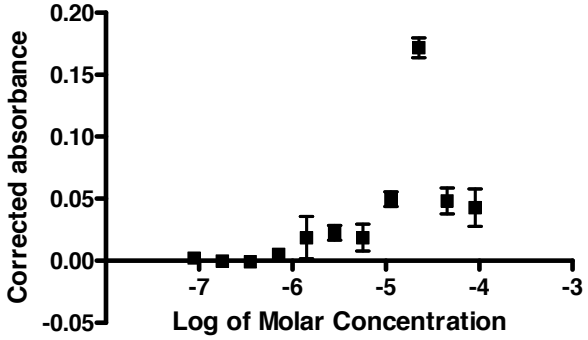


Figure D.8 Cytotoxic Dose Response Curve for 2-bromoacetamide in the YES Assay

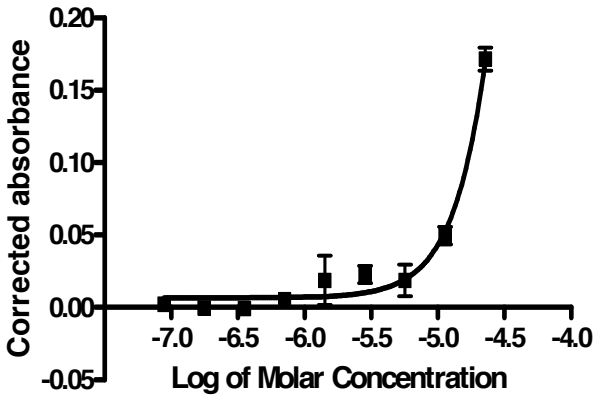


Figure D.9 Dose Response Curve for 2-bromoacetamide in the YES Assay

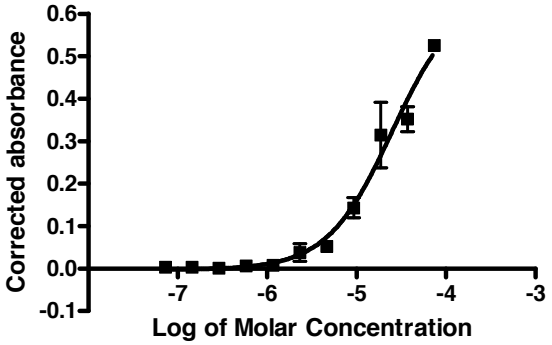


Figure D.10 Dose Response Curve for Mucochloric acid in the YES Assay

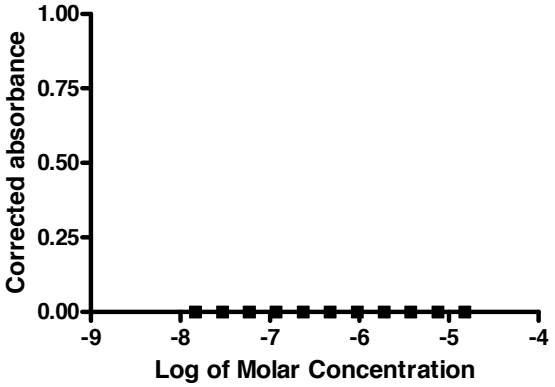


Figure D.11 Dose Response Curve for Chloral hydrate in the YES Assay

Table D.1 summarizes the EC_{50} and EC_{10} for the DBPs shown in figures D.1 to D.11. These values are comparable to those presented on page 67 in this thesis.

Table D.1 Summary of the EC_{50} and EC_{10} for Duplicate DBPs and Phenolic Compounds Analyzed

Compound	EC_{50} (M)	EC_{10} (M)
Dichloroacetonitrile	4.89×10^{-6}	1.52×10^{-6}
Mucochloric acid	2.49×10^{-5}	3.96×10^{-6}
2-bromoacetamide	1.68×10^{-4}	5.12×10^{-5}
Dibromoacetonitrile	4.63×10^{-5}	1.22×10^{-5}
Trichloroacetic acid	2.57×10^{-4}	6.98×10^{-5}
2, 4-dichlorophenol	1.62×10^{-4}	4.60×10^{-5}
Dibromoacetic acid	2.85×10^{-4}	1.52×10^{-4}
2-chlorophenol	1.05×10^{-5}	6.50×10^{-4}

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